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Reproductive Effort of Some Annual and Perennial Plant Species: Impact of Successional Sequence, Habitat Conditions and Plant Size

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Abstract. The reproductive effort of some annual and perennial plant species was investigated with respect to successional sequence, habitat conditions and plant size. In the psammosere succession (dune succession), the reproductive effort (RE) of *Cressa cretica* and *Atriplex griffithii* was significantly greater in the early stage compared to that in late succession. Likewise, in relation to lithosere succession, *Sporobolus arabicus*, *Pluchea lanceolata* and *Vernonia cenerescens* all showed high reproductive effort in early part of succession compared to that of late succession. The annuals (*S. arabicus* and *P. lancolata*) exhibited greater reproductive effort compared to the perennial species *Vernonia cinerescens*. Examination of the impact of site differences on reproductive effort showed that four grasses including *Setaria intermedia*, *Chloris barbata*, *Cenchrus biflorus*, and *Eragrostis pilosa* were found to have significantly ($P<0.05$) greater reproductive effort in site 1 (near cultivated field), compared to site 2 (a vacant lot), which had low nutrient level compared to site 1. The reproductive effort of *Sonchus asper* (a composite) did not exhibit significant difference between sites. The investigation of relationships between plant size (volume) and reproductive effort of *Solanum forskalii*, *Senna holosericea* and *Heliotropium ophioglossum* showed positive correlations between plant size and reproductive effort. *Solanum forskalii* and *Senna holosericea*, in particular, exhibited a close association in this respect. It is concluded that: 1) RE is greater in early compared to late succession, 2) RE changes with the habitat and 3) there seems to be a direct relationship between RE and plant size.

Keywords: reproductive efforts, succession, sand dunes, soil analysis, plant-size

Introduction

The survival of an individual (a genet) in a population is determined by a unique combined set of habitat conditions and life-history traits. Temporal pattern of its growth and reproduction is regulated by a variety of features including its growth rate, size, vegetative offspring (if any), the propagules produced per generative episode and the number of such reproductive events that occur during the life-history (Roff, 1992; Stearns, 1992). Each individual is subjected to the force of natural selection, both under the influence of habitat conditions that usually change with time as well as inter- and intra-specific competition. These limiting conditions act as the driving force for the plant to adapt to such life-history traits that would result in fitness (e.g., survival) of the individual and are collectively termed as life-history strategy.

One important aspect of the life-history of plant species in various environments is the resource allocation, i.e., how plants allocate their resources (energy) to different organs and functions (Harper, 1977; Harper and Ogden, 1970) with varied environments (Reekie and Bazzaz, 2005; Lovett-Doust, 1990) in particular to the reproduction (De Jong and Klinkhamer, 2005). The cost of reproduction is generally high which is expressed in the form of reduced growth rate and/or increased death rate (Reekie and Bazzaz, 2005; Bazzaz *et al*., 1979). The cost of reproduction is also presented as a compromise in the allocation of resources, i.e., current reproduction has an adverse effect on future reproduction and plant growth (Medez and Obesso, 1993; Reekie and Bazzaz, 1992; Fox and Stevens, 1991). Antflinger and Wendel (1997) pointed out the role of inflorescence as a source and sink for carbon assimilation that may lower the cost of reproduction and support frequent inflorescence production thus contribute to increased reproductive effort. Ashman (1994) suggested "dynamic" estimates of the cost of reproduction which is a function of reproductive photosynthesis or respiration, nectar production, or reproductive nutrient resorption to assess

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current reproductive investment in order to predict future reproductive effort. The inconsistent evidence for trade-offs between current and future reproduction has prompted much debate regarding the cost of reproduction and the methodology involved for testing it (Bailey, 1992).

Many facets of plant reproduction, such as seed size and number and reproductive potential (Salisbury, 1942) have long been the subject of focus for ecologists. In recent years, a great deal of work has been undertaken on the determination of reproductive effort, the proportion of total energy (biomass) allocated to reproduction in various plant species (Hancock and Pritts, 1987; Watson, 1984; Abrahamson and Caswell, 1982; Soule and Werner, 1981; Bazzaz *et al*., 1979; Harper and Ogden, 1970) and the allocation of energy to seed production. Among the life-history traits, reproductive effort including floral display and gamete production have paramount effects on plant fitness. The environmental conditions under which reproduction occurs can lead to variation in reproductive effort including floral morphological structures, function and production of seeds (Clifford, 2011). The relative size of plants in a community, as influenced by soil moisture and nutrients, herbivory and competition, appear to be directly correlated with seed size and number (Watkinson and White, 1985; Inouye *et al*., 1980; Solbrig *et al*., 1980). A trade-off between seed size and number has been well recognized (Harper, 1977; Werner and Platt, 1976).

The relationships between environmental factors and resource allocation pattern expressed in different populations of a species have not been fully explored. In the late 1960's ecologists began to develop theories pertaining to evolutionary basis of resource allocation patterns of which the pioneering work was that of (Reekie and Bazzaz, 2005; Harper and Ogden, 1970). Life-history theory predicts that a short juvenile period and high reproductive effort should be favoured in adverse or stochastic environment, where the life span is unpredictable and mortality is age and size independent (Torang *et al*., 2010; Silvertown *et al*., 2001; Kozlowski, 1992; Stearns and Koella, 1986). By contrast, delayed reproduction and lesser reproductive effort should be an optimal strategy in more stable environment, where mortality declines with age or size (Torang *et al*., 2010). Harper *et al*. (1970) suggested that the intensity of competition is a function of habitat maturity and as a result, early successional species are exposed to low competitive stress and are primarily annuals with high reproductive effort, while species appearing in later part of a particular sere face greater competitive stress, are usually perennials and have lower reproductive effort (Newell and Tramer, 1978; Gadgil and Solbrig, 1972). High proportions of energy allocated to vegetative parts were thought to confer fitness in a long-term severe struggle for existence in resource-limited stable environments (McNaughton, 1975; Gaines *et al*., 1974; Abrahamson and Gadgil, 1973). Abrahamson (1975) studied the reproduction of *Rubus hispidus* L., in relation to habitats of a secondary succession and found that the total reproductive effort (sexual and vegetative reproduction) declined with increased maturity of the site. Some studies comparing reproductive effort of a single species under different environmental conditions have shown contradictory results (Holler and Abrahamson, 1977; Van Andel and Vera, 1977; Werner and Rioux, 1977). In accordance with Hancock and Pritts (1987) two hypotheses have been tested with regard to the association of reproductive effort and successional maturity: 1) reproductive effort declines as succession progresses and 2) perennials have a lower reproductive effort than annuals. They found significant negative trend between reproductive effort and successional progression. Abrahamson (1979) showed that the species from early succession had higher reproductive effort than did herbs from late succession. Roos and Quinn (1977) found that early successional field population of *Andropogon scoparius* had a higher reproductive effort and a shorter developmental time (as indicated by dates of first anthesis) than populations of older successional field. Swamy and Ramakrishnan (1988) found that in late succession reproductive effort of *Mikania micrantha* was lesser compared to that of early succession. Comparing the annuals against the perennial with respect to their reproductive effort, Hancock and Pritts (1987) found that annuals had consistently greater reproductive effort than the perennials. Ploschuk *et al*. (2005) stated that seed yield and allocation to reproduction are actually lower in several perennial weeds compared with closely related annual crops. Similarly, Djordjevic-Miloradovic (1997) examining the changes in reproductive effort of *Tussilago farfara* populations demonstrated similar results. Silvertown and Dodds (1996) concluded that annuals have greater reproductive allocation compared to perennials. Nonetheless, certain studies have shown greater allocation to reproduction in perennial crops (DeHaan *et al*., 2003; Pimm, 1997).

McNamara and Quinn (1977) compared the populations of the annual *Amphicarpum pushii* Kunth and found the reproductive effort to differ with sites which was explained on the basis of differences in micro-environments. They further stated that the apparent trend of differential allocation of reproductive biomass in relation to site conditions, could be adaptive for this fugitive species and could result either from phenotypic plasticity or may be due to local genetic differentiation. Tallowin (1977) working with *Festuca contracta* T. Kirk showed that the sexual reproductive effort declined significantly with increasing habitat severity, principally exposure to high winds. Reduced floret and seed production resulting from severe habitat conditions have also been reported in *Phleum alpinum* (Callaghan and Lewis, 1971). Li *et al*. (2005) investigated the reproductive effort of *Artemisia halodendron* in two contrasting habitats and reported that plants inhabiting the less eroded semi-fixed habitats (dunes) produced more flowering shoots, greater dry weight of flowering shoots, dry weight of seeds and reproductive effort than those inhabiting the more eroded mobile dunes. He *et al*. (2009) examined the reproductive effort of an annual plant *Corispermum elongatum* Bunge in two types of sandy habitats and found significant effect on the pattern of reproductive allocation. The resource allocated to reproduction was size-dependent and also affected by habitat types. Torang *et al*. (2010) tested among-population differentiation in reproductive effort of *Primula farinosa* L., using sites that differed widely in soil depth and water retaining capacity and found that reproductive effort varied among populations and negatively correlated with soil depth. Soule and Werner (1981) stated that the relationship between environmental conditions and the resource allocation patterns have not yet been fully worked out. This raises the need of further studies along this line.

Size-dependent variation in reproductive effort is now a fairly well known phenomenon. Size is a better predictor of reproductive status than age (Waugh and Aarssen, 2012; Hanzawa and Kalisz, 1993). It has been noticed that plants have to attain a minimum size below which they do not reproduce regardless of age (Lacey, 1986). Size-dependent variation in reproductive effort in plants has been theoretically predicted a long time ago (Gadgil and Bossert, 1970). Samson and Werk (1986) gave a simple model for the examination of the pattern of reproductive allocation. Their results suggested that much of the variation in reproductive effort can be explained on the basis of intrinsic size effects rather than extrinsic factors. However, in many studies of reproductive effort, size dependent effects have been completely ignored. Ohlson (1988) investigated the size-dependent reproductive effort in the populations of *Saxifraga hirculus*. In *S. hirculus* the probability of flowering increased with plant size, which has also been reported for some other species as well (Pritts and Hancock, 1983; Van der Meijden and Van der Waals-Kooi, 1979). Welham and Setter (1998) studied the size-dependent reproductive effort of *Taraxacum officinale* Weber populations and found that reproductive effort increased linearly with increasing vegetative biomass but the slope for the population from alfalfa field (disturbed site) was significantly greater than that derived from undisturbed sites with high grass density. Mendez and Obeso (1993) reported a linear relationship between reproductive allocation and plant size in *Arum italicum*. Mendez and Karlsson (2004) found reproductive biomass to be size-dependent in all the studied populations of *Pinguicula vulgaris* L. Likewise, Kawano and Mikaye (1983) working with five species of *Setaria* found greater fecundity and reproductive biomass above a threshold size to be size-dependent. Hartnett (1990) reported that the sexual reproductive effort in four clonal composites was a monotonically increasing function of ramet size. Pino *et al*. (2002) demonstrated a linear relationship between reproductive biomass and vegetative biomass in *Rumex obtusifolius* indicating a size dependent reproductive pattern.

The principal objectives of the present study were: 1) to investigate the impact of successional sequence on the reproductive effort of some annual and perennial plants, 2) to examine the effect of site differences on the reproductive effort of some plants and 3) to relate reproductive effort with plant size.

Materials and Methods

Study sites. All study sites were located in Karachi city, southern Sindh, Pakistan or its vicinity. The physiographic situations ranged from plains to sand dunes to calcareous hills around Karachi. The study sites were located within the greater Karachi. In all, 8 sites were sampled site 1=Sandspit, site 2=Clifton, site 3=Paradise point, site 4=Gdap, site 5=Cultivated field in Karachi university campus, site 6=Vacant lot near Dept., of Statistics, university of Karachi, site 7=Karachi university campus near Dept., of Physiology, university of Karachi, site 8=near Pipri. The soils were in general coarse

textured and poor in nutrients and organic matter. The soil from each site was collected with a soil auger (except at Paradise Point, where soil was very shallow) to a depth of 25 cm. Soils were analysed physically and chemically. Soil texture was determined using a set of sieves of various sizes. Soil pH was determined in a soil paste (1:5, soil: distilled water) using a Jenway pH meter (Model 3505). The characteristics of the soils pertaining to individual experiments are given in appropriate sections.

Effect of successional sequence. Reproductive effort of five species was investigated in two different series. One successional sequence was that of sand dune system prevailing at Sandspit and Clifton area in Karachi, southern Sindh, Pakistan. The unstabilized, mobile dunes at Sandspit (having sparse vegetation cover) can easily be distinguished from stabilized (fixed) dunes that have plenty of vegetation cover, particularly that of *Ipomoea pescaprae* L. R. Br., a dominating species in the late psamosere succession. *I. pescaprae* horizontally spreads on the dunes profusely (stems trailing, rooting at the nodes), binds the sand and prevents it from moving in bulk. Such dunes are situated in Clifton area though most of them have now been destroyed due to rapidly growing construction work. The plant species chosen for the study were *Atriplex griffithii* Moq and *Cressa cretica* L., both being perennial halophytic under-shrubs occur sympatrically. The dune vegetation at Sandspit is dominated by *Suaeda fruticosa* Forssk, *Salsola imbricata* Forssk., *Salvadora persica* Linn., *Atriplex griffithii*, *Cressa cretica*, *Zygophyllum simplex* Linn., and *Ipomoea pescaprae* as well as grasses such as *Halopyrum mucronatum (*Linn.) Stapf, *Aeluropus lagopoides* (Linn.) Trin., *Urochondra setulosa* (Trin) C.E. Hubbard and a sedge *Cyperus conglomeratus* subsp., *curvulus* (Boeck) Kukkonen. The vegetation at Clifton area (undisturbed permanent dunes) is dominated by *Ipomoea pescaprae*, *Suaeda fruticosa*, *Salsola imbricata*, *Heliotropium bacciferum* subsp. *lignosum* (Vatke) Kazmi, *Cressa cretica* (all shrubs or undershrubs) and grasses including *Dichanthium annulatum* (Forssk) Stapf., *Lasiurus hirsutus* (Forssk) Boiss and *Aeluropus lagopoides*.

The other successional sequence was a lithosere associated with calcareous hills near Paradise Point and Gadap area. The species investigated were two annuals namely *Sporobolus arabicus* Boiss and *Pluchea arguta* Boiss and a perennial species *Vernonia cinerescens* Schultz-Bip. The vegetation at Paradise Point which represented early succession consisted of under-shrubs such as *Iphiona grantioides* (Boiss) Anderb, *Ruellia patula* Jacq., *Ruellia longifolia* (Stocks) T. Anders, *Barleria acanthoides* Vahl and *Pulicaria hookeri* Jafri and grasses such as *Chrysopogon aucheri* (Boiss) Stapf, *Cymbopogon jwarancusa* (Jones) Schultes and, *Sporopbolus arabicus*, etc. Whereas, the vegetation at Gadap area representing late succession was dominated by *Commiphora wightii* (Am) Bhandari, *Grewia tenax* (Forsk) Fiori, *Euphorbia caducifolia* Haines, *Vernonia cinerescens* SchBip, *Grewia villosa* Willd. and *Acacia senegal* (L) Willd. In this mature type of vegetation both *Sporobolus arabicus* and *Pluchea arguta* are infrequent. *S. arabicus* often occurs in abundance after the monsoon rains. At least five plants of each of the selected species were randomly collected from the study sites. Plants included the roots and the underground parts (if any) and placed in between blotting papers, kept in plant presses and brought to the laboratory. The plants were split into vegetative and reproductive parts (flowers, fruits, seeds and peduncle if present) oven dried at 75 °C for 24 h and weighed. The reproductive effort (RE) was determined as RE=(reproductive weight / total dry weight) × 100.

Data on reproductive effort were subjected to analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) test, performed at a rejection probability level = 0.05.

Effect of habitat conditions. Five sympatrically occurring species were chosen for this study of which four were grasses, namely *Setaria intermedia* Roem. & Schult., *Chloris barbata* Swartz, *Cenchrus biflorus* Roxb., *Eragrostis pilosa* (L) P. Beauv and one annual Composite *Sonchus asper* (L) Hill. All of these species occurred in a cultivated field in Karachi university campus where vegetables are grown, served as one habitat (site 1). Except for *Sonchus asper*, that grows in winter, the grasses were sampled during summer. The other sampling site for these species was located in a vacant lot (site 2) near Statistics Department, university of Karachi where all the six species were found and sampled. The plants were chosen randomly except *Sonchus asper* which was infrequent and had to be sampled deterministically. The entire plants including underground parts were collected. The rest of the procedure was the same as outlined above. The soil samples from both sites were also collected and analysed physically and chemically in accordance with the procedures mentioned earlier.

Effect of plant size. Three perennial species were chosen *Solanum forskalii* Dunal, *Senna holosericea* (Fresen) Greuter and *Heliotropium ophioglossum* Stocks ex Boiss., *Solanum forskalii* population was located at Karachi university campus (near department of Physiology) and it was almost a pure population so that intraspecific competition was greater than interspecific competition (Shaukat *et al*., 2009). The population of *Senna holosericea* and *Heliotropium ophioglossum* were located near Pepri (about 35 km from Karachi city on National Highway) and the two species occurred sympatrically. Circumference was measured of thirty plants of each species, and the volume of each plant was calculated as a cylindrical shaped structure (Shaukat *et al*., 2012; Shaltout *et al*., 2003). The entire plants, together with roots were collected and brought to laboratory in polythene bags. The reproductive effort was determined as described above. Product moment correlation coefficients were calculated and linear regressions was performed between plant size (volume) and the reproductive effort. All programs including DISPERS (for computation of mean and SE), ANOVA to compute analysis of variance and the post-hoc test "least significant difference" LSD, and CORREG to perform correlation and linear regression were written by the first author (SSS) in C++ and are available on request. Similar programs written in BASIC are also available by Ahmed and Shaukat (2012).

Results and Discussion

Effect of successional sequence. The soil characteristics of early and late dune succession are given in Table 1. The soils of both the successional sequence are extremely sandy with low water retaining capacity. The soils of late succession at Clifton area have greater organic matter and slightly greater levels of nutrients. Salinity is greater at Sandspit presumably because of nearness of sea and the salt spray. The reproductive effort of the five species in relation to successional sequence is shown in Table 2. The two species chosen for the psamosere (sand dune) succession showed greater reproductive effort for early succession compared to late succession. *Cressa cretica* responded to successional sequence more than did *Atriplex griffithii* showing greater disparity between early and late successional stages. It is possible that the reproductive effort of *A. griffithii* is slightly underestimated because of the loss of seeds as a consequence of wind dispersal. Wind speed at the coast is in general high and the seeds of *A. griffithii* are anemochoric.

Table 1. Soil characteristics of unstabilized shifting and fixed sand dunes at Sandspit and Clifton respectively, Mean ± Standard Error (SE)

Soil characteristics	Unstabilized dunes	Fixed dunes
Coarse sand %	57.24±2.15	45.36±1.85
Fine sand %	40.53±1.52	49.54±1.77
Silt and clay %	2.23±0.31	5.10±0.84
Water holding capacity	5.20	8.04
pH	8.2	7.9
Organic matter %	0.280	0.438
Total Kjeldahl nitrogen	0.58	0.088
Exchangeable Ca	44	62
Exchangeable Mg	5.0	9.0
Electrical conductivity EC (μs/cm)	20	18

Table 2. Mean and range and SE (standard error) of reproductive effort of species in early and late psamosere and lithosere succession, Rows means not sharing the same letter are significantly different(P=0.05)

Sere/Species	Early succession		Late succession	
	Range	Mean ± SE	Range	Mean ± SE
Psamosere				
Atriplex griffithii	8-19	12.8±1.4[a]	6-11	8.2±0.8[b]
Cressa cretica	10-24	17.4±1.6[a]	8-17	11.5±1.7[b]
Lithosere				
Sporopolous arabicus	18-26	21.2±1.27[a]	12-21	17.5±1.24[b]
Pluchea arguta	16-27	23.5±1.47[a]	9-19	15.3±0.85[b]
Vernonia cinerescens	13-21	16.7±0.87[a]	10-15	12.4±0.92[b]

The soil properties of the hill slopes of early and late succession are given in Table 3. The soil texture in the late succession is slightly finer with greater percentage of silt plus clay and consequently has greater maximum water holding capacity. Organic matter % and the concentrations of exchangeable Ca, Mg and K were also at a higher level in late succession compared to early succession. The reproductive effort of all three test species was higher in the early succession compared to late lithosere succession. The annuals namely *Sporobolus arabicus*, an annual grass and *Pluchea arguta*, an annual forb showed high reproductive effort 21.2 and 23.5, respectively. The perennial species *Vernonia cinerescens* also showed high reproductive effort in the early lithosere succession. Compared to the reproductive effort of psamsere progression the reproductive effort was higher for species in the lithosere succession. Hancock and Pritts (1987) concluded that

Table 3. Soil characteristics of calcareous hills at early (Paradise Point) and late succession (Gadap)

Soil characteristics	Early succession	Late succession
Coarse sand %	51.8 ± 1.27	38.2
Fine sand %	28.7 ± 1.47	33.5
Silt and clay %	19.5 ± 0.82	28.3
Water holding capacity	20.6	28.3
pH	7.8	7.6
Organic matter %	0.341	0.568
Total Kjeldahl nitrogen	0.210	0.431
Exchangeable Ca	52 ± 2	61 ± 3
Exchangeable Mg	28 ± 2	32 ± 2
Exchangeable K	18 ± 1	26 ± 3

reproductive effort in general declines with the onward progression of succession. Harper *et al*. (1970) suggested that intensity of competition is correlated with habitat maturity and as a consequence, early successional species, which are primarily annuals, have large reproductive effort, while species dominating the later part of sere, which are usually perennials, have smaller reproductive effort. The changes in reproductive effort in the present study during psamosere contrasts with the findings of He *et al.* (2009) who found greater reproductive effort of *Corispermum elongatum* growing on fixed or stabilized dunes compared to that on mobile or embryonic dunes. On the other hand, present study accords with the findings of Gleeson and Tilamn (1990) who found that the reproductive biomass declined during succesional progression. Likewise, Swamy and Ramakrishnan (1988) found that the reproductive effort of *Mikania micrantha* was greater in 3 year old field compared to that of 12 years fallow field. In later succession, plants showed adaptation for survival and competition and also increased allocation to rosette and root because they serve as perennating organs. Roos and Quinn (1977) reported that in early successional field population of *Andropogon scoparius* had higher reproductive effort and a shorter developmental time than population of older site. Djordjevic-Miloradovic (1997) working on the reproductive effort of *Tussilago farfara* growing on coal ash and found greater reproductive effort of *T. farfara* in early succession (1-2 years) compared to 8-9 years old population. In the mature stage of succession, plants exhibited adaptations to vegetative way of reproduction.

The annual species *Sporobolus arabicus* and *Pluchea arguta* exhibited greater reproductive effort compared to the perennial species *Vernonia conerescens.* Annuals generally colonize in early succession in heterogeneous, unpredictable and xeric environments and exposed to low competitive stress, therefore, they devote more energy to reproduction. Ploschuk *et al*. (2005) demonstrated greater allocation to reproduction in annual compared to perennial *Lesquerella* crop. Usually perennials which face greater competitive stress, as they predominate the mid and later succession, have lower reproductive effort (Newell and Tramer, 1978; Gadgill and Solbrig, 1972).

Influence of habitat. The soil analysis of the two study sites (cultivated field, site 1 and vacant lot, site 2) are presented in Table 4. The soil was sandy loam at site 1 while it was loamy sand at site 2. The proportion of silt + clay was considerably higher at site 1. Likewise, the soil of site 1 also had greater percentage of organic matter and the nutrients. In particular, nitrogen percentage was significantly ($P<0.05$) higher in the arable field soil. All the grass species including *Setaria intermedia*, *Chloris barbata, Eragrostis pilosa* and *Cenchrus biflorus* were found to have significantly ($P<0.05$) greater reproductive effort in site 1 compared to site 2 (Table 5). However, a dicot weed *Sonchus asper* did not show a significant difference in the reproductive effort between the two sites. It is apparent that in most of the species tested the reproductive effort declined significantly with the habitat severity. The cultivated field undoubtedly having better moisture regime due to irrigation and better nutrient regime owing to fertilizer application as well as good aeration afforded to the roots resulted in enhanced reproductive effort to increase the population size. Although, it must be borne in mind that in this

Table 4. Soil characteristics of two sites at Karachi University campus (cultivated field (Site 1) and vacant lot (Site 2)

Soil characteristics	Site 1 (near field)	Site 2 (vacant lot)
Coarse sand %	41.4	44.8
Fine sand %	31.5	32.4
Silt and clay %	27.1	22.8
Water holding capacity	32.2	25.2
pH	7.7	7.7
Organic matter %	0.853	0.437
Total Kjeldahl nitrogen	0.430	0.240
Exchangeable Ca	78±3	50±2
Exchangeable Mg	32±2	27±2
Exchangeable K	25±1	19±2

Table 5. Range and mean reproductive effort of 5 replicate plants of each species. Means followed by a different letter in a row are significantly different at P=0.05

Species	Site 1		Site 2	
	Range	Mean	Range	Mean
Setaria intermedia	9-15	13.77±0.79[a]	8-12	9.7±0.72[b]
Chloris barbata	12-19	15.2±1.2[a]	11-15	12.0±0.69[b]
Cenchrus biflorus	25-33	31.7±1.3[a]	22-30	26.4±1.1[b]
Eragrostis pilosa	18-27	24.5±1.4[a]	16-23	18.8±1.3[b]
Sonchus asper	15-21	18.6±0.92[a]	11-19	16.9±1.2[a]

habitat competition is likely to be more severe because of the crop. It is observed that these grasses or weeds generally do not grow in close neighbourhood of the crop plant. Moreover, the grasses tested here usually grew near the periphery of the field thereby avoiding direct competition from the crop plant but take advantage of better growth conditions of the cultivated field. On the other hand the same grasses when growing sympathitically in the semi-natural community (Site 2) are subjected to interspecific competition. Also they are subjected to feeding activity of phytophagous insects and exposed to high wind velocity (3-4 miles/h). Besides, competitive stress could be an important factor responsible for decreased reproductive effort under the condition of co-occurrence of a number of species including the grasses and other perennial herbs and shrubs. Tallowin (1977) examined the sexual reproductive performance of *Festuca contracta* (T. Kirt) demonstrated that seed production declined with increasing habitat severity. Reduced floret and seed production because of severe habitat conditions has also been found in another grass *Phleum alpinum* (Callaghan and Lewis, 1971). Li *et al.* (2005) studied the reproductive effort of *Artemisia halodendron* in two contrasting habitats. Plants growing on the less eroded semi-fixed habitats (dunes) produced greater number of flowering shoots, higher dry weight of flowering shoots, seed dry weight increased reproductive effort than those inhabiting the more eroded mobile dunes, thereby demonstrating the negative effect of habitat severity. The results of the current study suggest that the between population variation in reproductive effort in relation to site conditions observed in the field largely reflects phenotypic plasticity in response to local environmental conditions. Phenotypic plasticity with respect to reproductive effort has also been reported for *Polygonum cascadense* by Hickman (1975). On the other hand, local genetic differentiation can be ruled out as this process is favoured by selection when spatial differences in environmental conditions are consistent over time and seed dispersal between habitats is limited. In the present study the two habitats were not too far from each other (about 300 m) and gene exchange was highly likely to occur between populations (in fact the two populations can be regarded as part of a metapopulation) and both the habitats may be categorized as temporary and disturbed, thereby restricting the chances of long-term selection pressure.

Effect of plant size. The soil characteristics of the two sites namely Site 1 (Karachi university campus near department of Physiology) and site 2 (Pepri 35 km from Karachi city) are given in Table 6.

Table 6. Soil characteristics of two sites where plants size and RE studies were performed

Soil characteristics	Karachi university	Pepri
Coarse sand %	72.6	70.1
Fine sand %	16.1	16.3
Silt and clay %	11.3	13.6
Water holding capacity	29.2	30.8
pH	7.8	8.0
Organic matter %	0.35	0.32
Exchangeable Ca	22±2	50±2
Exchangeable Mg	49±2	62±3
Exchangeable K	25±1	30±2
Available PO_4	16	24
Total Kjeldahl nitrogen	0.38	0.42

The relationships between plant size (volume) and reproductive effort for the three species, namely *Solanum forskalii*, *Senna holosericea* and *Heliotropium ophioglossum* are shown in Fig. 1-3, respectively. Product moment correlation coefficients were calculated between plant size (volume) and reproductive effort (RE). All three species showed significant positive correlation between plant size and reproductive effort: *Solanum forskalii* r=0.721 (P<0.001), *Senna holosericea* r=0.749 (P<0.001), *Heliotropium ophioglossum* r=0.0.443 (P<0.05). The range of variation in RE varied among the species. Reproductive effort ranged between 9.2 to 20.1% for *Solanum forskalii*, 8.2 to 17.3% for *Senna holosericea* and 5.7 to 16.4% for *Heliotropium ophioglossum*. The regression equations between plant size (PS) and

reproductive effort (RE) are give below for the three species:

Solanum foskalii
PS=10.502+0.0134 RE R^2=52.0% R^2 adj=50.3%
Senna holosericea
PS=8.331+0.0161 RE R^2=56.2% R^2 adj=54.6%
Heliotropium ophioglossum
PS=10.201+0.0103 RE R^2=19.6% R^2 adj=16.7%

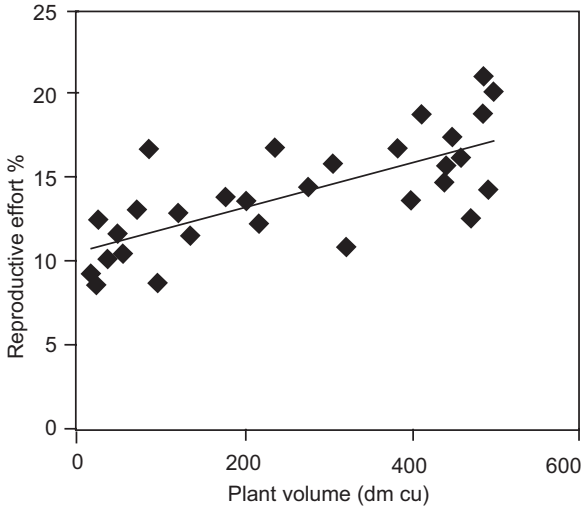

Fig. 1. Relationship between size of *Solarum faskalii* individuals and % reproductive effort. Linear regression line is fitted to the data, R^2=52.0%.

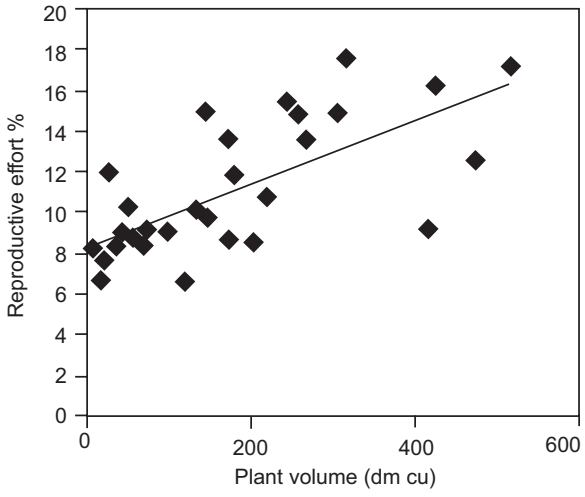

Fig. 2. Relationship between size of *Senna holosericea* individuals and % reproductive effort. Linear regression line is fitted to the data, R^2=56.2%.

High values of coefficient of determination (R^2) indicate that most of the variation in reproductive effort is the result of variation in the plant size (volume). Apart from linear regression, other forms of regression were also tried. Linear relationships provided better fits to the observed data than logarithmic, power or exponential models for each of the species.

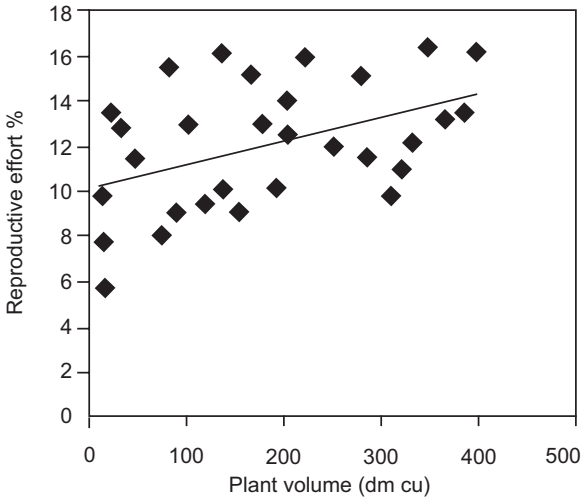

Fig. 3. Relationship between size of *Heliotropium ophioglossum* individuals and % reproductive effort. Linear regression line is fitted to the data, R^2=19.6%.

Size dependent variation in reproductive effort has been demonstrated in a number of empirical investigations (Ohlson, 1988; Kawano and Masuda, 1980; Gaines *et al*., 1974; Abrahamson and Gadgil, 1973). Usually the reproductive effort increases with increasing plant size (Mendez and Karsson, 2004; Aarssen and Jordan, 2001; Welham and Setter, 1998; Schimid abd Weiner, 1993; Hartnett, 1990). Good direct evidence of the pattern of size-dependent reproductive effort and linear relationships between reproductive biomass and vegetative biomass in some perennial species has been provided by Weaver and Cavers (1980) and Waite and Hutchings (1982). On the other hand, Shipley and Dion (1992) found no evidence for size-dependent reproductive effort in a number of herbaceous species. Ohlson (1988) showed that in site with low pH, low nutrients and water supply no relationship existed between seed production and ramet size. However, under favourable site conditions fecundity was directly correlated with the ramet size. Nonetheless, the relationships between RE and size have not been studied under semi-desert

or desert conditioned for the under-shrubs. Furthermore, it is noteworthy that the relationship between plant size (volume) and reproductive effort is highly significant for all three species investigated in our study as shown by high magnitudes of correlation coefficient. These results corroborate the findings of Aarssen and Jordan (2001). All three plant species tested in this study were under-shrubs and not much is known regarding the relationship between size and reproductive effort in these life-forms. The results of isometric relationship parallels the results of an earlier within species study which disclosed that reproductive output per unit plant size is constant across plants of different sizes when size variation is predominately controlled by environmental variation and plants are harvested at final stage of development (Clauss and Aarssen, 1994). A large plant obviously has greater resources to support high fecundity (and/or large seed size) and therefore, high reproductive effort. The limitation of the current study is that it is a snapshot of the situation with regard to the magnitude of reproductive effort as it based on a single year of field observations. In those studies where workers have considered between year variations it has been found that that RE varies from year-to-year (Ohlson, 1988; Soule and Werner, 1981). More importantly, the relationship between RE and plant size can change from year-to-year (Ohlson, 1988). Thus, it is recommended that, in order to study the relationship between RE and plant size, the study should be conducted for at least three years at different sites ranging from nutrient and moisture austerity to high nutrient and moisture regimes or along environmental gradients.

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Amino Acids Profile of the Fancy Meats of the African Giant Pouch Rat (*Cricetomys gambianus*)

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Abstract. The levels of amino acids were determined in the tongue, liver, kidney and heart (fancy meats) of African Giant pouch rat (*Cricetomys gambianus*) on dry weight basis. The results showed that the total essential amino acids ranged from 40.2-43.8 g/100 g crude protein or 46.4-48.4% of the total amino acids. The amino acid scores showed that Lys ranged from 0.97-1.12 (on whole hen's egg comparison), 1.10-1.27 (on provisional essential amino acid scoring pattern), and 1.04-1.20 (on suggested requirement of the essential amino acid of a pre-school child). The predicted protein efficiency ratio was 2.15-2.62, the essential amino acid index range was 1.14-1.31 and the calculated isoelectric point range was 4.82-5.22. The chi square (X^2) test was low and none of the parameters were significantly different at $\alpha = 0.05$ on all the comparisons made. Results had good comparison with whole hen's egg protein and other standard proteins.

Keywords: red viscera, amino acids profile, *Cricetomys gambianus*

Introduction

The Gambian pouch rat (*Cricetomys gambianus*), also known as the African Giant pouch rat is a nocturnal rat of the giant pouch rat genus *Cricetomys*. It is the largest muroid in the world. It is native to Africa (Oyarekua and Adeyeye, 2011).

The Gambian pouch rat can grow to be as big as a raccoon and can weigh up to 4 kg. It has very poor eyesight and so depends on its senses of smell and hearing. Its name comes from the large, hamster-like pouches in its cheeks. It is not a true rat, but is part of a uniquely African branch of muroid rodents. In its native Africa, this rat lives in colonies of up to twenty, usually in forests and thickets, but also commonly in termite mounds. It is omnivorous, feeding on vegetables, insects, crabs, snails, and other items, but apparently preferring palm fruits and kernels (Novak and Paradiso, 1991).

Unlike domestic rats, it has cheek pouches like a hamster. These cheek pouches allow it to gather up several kilograms of nuts per night for storage underground. It has been known to stuff its pouches so full of date palm nuts so as to be hardly able to squeeze through the entrance of its burrows. The burrow consists of a long passage with side alleys and several chambers, one for sleeping and the others for storage (Oyarekua and Adeyeye, 2011). The African Giant pouch rat belongs to the order Rodentia, superfamily Muroidea, family Nesomyidae, subfamily Cricetomyinae, genus *Cricetomys,* species *gambianus*, Binomial name: *C. gambianus* Waterhouse, 1840 (Oyarekua and Adeyeye, 2011). In Africa, it is routinely eaten as bush meat.

A study carried out in Nigeria, showed that the Giant rat produces about the same amount of meat as the domestic rabbit (Den Hartog and de Vos, 1973) and its nutritional value has been compared favourably with that of domestic livestock. African villagers know how to prepare it by smoking or by salting (NRC, 1991). Oyarekua and Adeyeye (2011) have reported the amino acids profile of its brain and eyes.

In many developing countries meat animals are frequently slaughtered only for the carcass, whereas, a number of by-products, which can be obtained quite easily, could help to improve the supply of low-cost high-protein foods for people. They are generally consumed either as main ingredients in traditional dishes or as ingredients in meat products. The so-called red viscera: liver, heart, kidneys, tongue and neck sweetbread (thymus) are normally obtained and marketed as "fancy meats" (Fornias, 1996), who has reported the main characteristics (recovery, preparation, microbiology, shelf-life and utilisation of edible by-products) in meat products. The African giant pouch rat is a delicacy in Nigeria, however, no literature report is available on the amino acids profile of its red viscera. This work

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was therefore, set out to evaluate the amino acids profile of the tongue, liver, kidney and heart of the African Giant pouch rat. The information derived here would also likely improve the information on different food compositions.

Materials and Methods

Sample collection and treatment. Matured female *C. gambianus* were caught in the wild by a local hunter commissioned for the purpose at Iworoko Ekiti, Nigeria, identified and immersed in hot water for 10 min. After hair removal the animals were dissected and the red viscera was separately removed and dried to constant weight then milled into flour and kept in freezer in McCartney bottles for analysis.

Crude protein determination and fat extraction. The micro-Kjeldahl method as described by Pearson (1976) was followed to determine the fat-free crude protein. The fat was extracted with a chloroform/methanol (2:1 v/v) mixture using Soxhlet extraction apparatus (AOAC, 2005).

Amino acid analysis. About 30 mg defatted samples were weighed into glass ampoule, 7 mL of 6 M HCl added and hydrolysed in an oven preset at 105±5 °C for 22 h. Oxygen was expelled in the ampoule by passing nitrogen gas into it. Amino acid analysis was done by ion-exchange chromatography (Spackman *et al*., 1958) using a Technicon Sequential Multisample Amino Acid Analyser (Technicon Instruments Corporation, New York, USA). The period of analysis was 76 min, with a gas flow rate of 0.50 mL/min at 60 °C, and the reproducibility was ± 3%.

Estimation of isoelectric point (pI). The theoretical estimation of isoelectric point (pI) was determined using the equation of Olaofe and Akintayo (2000) and information provided by Finar (1975):

$$IPm = \sum_{i=1}^{n} IPiXi$$

where:

IPm = isoelectric point of amino acid, IPi = isoelectric point of the i^{th} amino acid in the mixture and Xi = mass or mole fraction of the i^{th} amino acid in the mixture (Olaofe and Akintayo, 2000).

Estimation of predicted protein efficiency ratio (P-PER). The predicted protein efficiency ratio (P-PER) was estimated by using the equation of the form (Alsmeyer *et al*., 1974):

P-PER = -0.468 +0.454 (Leu) -0.105 (Tyr).

Estimation of dietary protein quality. The amino acid scores were calculated using three different procedures:

(i) scores based on amino acid values compared with whole hen's egg amino acid profile (Paul *et al*., 1976);
(ii) scores based on essential amino acid scoring pattern (FAO/WHO, 1973);
(iii) scores based on essential amino acid suggested required pattern for preschool child (FAO/WHO/UNU, 1985).

Estimation of essential amino acid index (EAAI). The essential amino acid index (EAAI) was determined using the method of Steinke *et al.* (1980).

The leucine/isoleucine ratios. The leucine/isoleucine ratio, their differences and their percentage differences were calculated.

Statistical analysis. The coefficients of variation per cent (CV%) were calculated for the parameters investigated. Also calculated was the chi square (X^2) test for all the parameters and subjected to table standards to test for significance difference, the level of probability was set 0.05 at n-1 degrees of freedom (Oloyo, 2001).

Results and Discussion

Amino acids composition of the samples has been presented in Table 1. Glu and Asp were the most concentrated amino acids (AA) in all the samples. Tryptophan was not determined. Table 1 shows that the essential amino acid (EAA) of the samples were mostly concentrated (on pair wise comparisons) in the liver; the trend was: Lys, Leu, Ile, Thr and Val (five EAA, 5/9 or 55.6%) in liver; Arg and Phe (two EAA, 2/9 or 22.2%) in tongue; His and Met (two EAA, 2/9 or 22.2%) in kidney. The coefficient of variation per cent (CV%) ranged between 4.20-28.9 in the AA, with Arg having the least CV%. Table 2 shows the EAA together with Cys and Tyr for the heart, kidney, liver and tongue of cattle, pig and sheep as observed by Fornias (1996). With these literature values, the present results could be said to be very favourably comparable to them, as (g/100 g): His (2.30-3.13); Thr (2.60-4.20); Val (3.64-5.60); Met (2.16-2.55); Ile (3.65-4.20); Leu (6.50-6.94); Phe (4.11-4.80); Tyr (3.17-3.65) and Cys (0.88-1.20). The comparisons showed that His and Met were better

concentrated in the African giant pouch rat than in cattle, pig and sheep red viscera, whereas, the present samples were also better than the minimum in Phe, Tyr and Cys. Total EAA from literature for cattle, pig and sheep red viscera were (g/100 g) as shown in Table 2 (including Try), whereas, the present results (without Try which was not determined in this work) had heart (36); kidney (34.7); liver (42.2) and tongue (37.4), which were all favourably comparable to the literature values, respectively.

Table 1. Amino acid composition of fancy meats of African giant pouch rat (dry weight)

Amino acid	Tongue	Liver	Kidney	Heart	CV %
	(g/100 g crude protein)				
Lys	6.75	6.96	6.37	6.03	6.31
His	2.32	2.30	3.13	3.03	16.6
Arg	6.99	6.38	6.81	6.50	4.20
Asp	10.6	9.70	10.5	10.6	4.21
Thr	2.60	4.20	3.95	2.65	25.2
Ser	3.95	3.72	3.55	3.00	11.4
Glu	15.2	13.0	14.2	12.9	7.89
Pro	2.87	4.22	3.20	3.50	16.7
Gly	4.85	8.00	6.20	4.20	28.9
Ala	4.48	4.18	3.94	4.07	5.53
Cys	0.88	1.20	1.05	1.20	14.1
Val	3.64	5.60	4.20	5.02	18.8
Met	2.38	2.16	2.55	2.40	6.77
Ile	4.02	4.20	3.65	3.65	7.10
Leu	6.70	7.65	6.50	6.94	7.22
Tyr	3.65	3.65	3.17	3.49	6.48
Phe	4.80	4.30	4.11	4.19	7.13
Protein (fat free)	70.3	86.9	80.5	86.3	9.50

The FAO/WHO/UNU (1985) EAA standards for pre-school children between 2-5 years (g/100 g protein) are also shown in Table 2. Based on this information, the tongue would provide enough or even more than enough of Leu, Phe +Tyr, Val, Ile, Lys, Met + Cys, His and total EAA, this trend was followed by EAA in the heart; liver satisfied all the requirements, whereas, kidney satisfied all the requirements except in Leu (6.50 < 6.6 g/100 g). Histidine is a semi-essential amino acid particularly useful for children growth. It is the precursor of histamine present in small quantities in cells. Methionine is an EAA with value range of 2.16-2.55 g/100 g cp in this report or 3.26-3.60 g/100 g cp with Cys. Methionine is needed for the synthesis of choline. Choline forms lecithin and other phospholipids in the body. When the diet is low in protein, for instance in alcoholism and kwashiorkor, insufficient choline may be formed; this may cause accumulation of fat in the liver (Bingham, 1977). Phenylalanine formed a value range of 4.11-4.80 g/100 g cp of the samples. It is the precursor of some hormones and the pigment melanin in hair, eyes and tanned skin. Phenylketonuria is the commonest inborn error of metabolism which can be treated successfully by diet. The absence of an enzyme

Table 2. Essential amino acid composition of fancy meats of cattle, pig, sheep and FAO/WHO/UNU (1985) standards for preschool children

Amino acid	Tongue			Liver			Kidney			Heart			
	C[a]	P[b]	S[c]	C	P	S	C	P	S	C	P	S	FAO/WHO/UNU
	(g/100 g crude protein)												
Lys	7.7	8.2	7.1	6.9	7.7	5.4	6.6	7.2	6.5	8.2	8.3	7.5	5.8
Met +Cys	3.4	2.2	3.2	4.0	4.4	3.1	2.9	4.3	3.1	3.9	4.4	3.0	2.5
Ile	4.3	4.6	3.9	4.6	5.1	4.3	4.1	5.3	4.0	4.4	4.8	4.3	2.8
Val	4.8	5.2	4.8	6.2	6.2	5.5	6.2	6.0	5.9	5.2	5.3	5.0	3.5
Tyr	0.8	1.2	1.0	1.4	1.4	1.2	1.4	1.3	1.4	1.1	1.2	1.1	1.1
Thr	4.4	4.2	4.5	4.6	4.2	4.5	4.8	4.1	4.7	4.7	4.4	4.7	3.4
Phe +Tyr	7.3	4.1	6.6	9.3	8.3	8.1	8.6	8.3	8.2	8.1	7.8	7.4	6.3
Leu	7.5	8.0	7.1	9.4	8.9	8.2	8.0	9.0	7.5	8.8	9.0	8.5	6.6
His	2.6	2.5	2.2	2.7	2.7	2.4	2.6	2.5	2.6	2.7	2.5	2.3	1.9
Total	45.8	40.2	40.4	49.1	48.9	42.7	45.2	48.0	43.9	47.1	47.7	43.8	33.9

[a] = cattle; [b] = pig; [c] = sheep.

in the liver blocks the normal metabolism of phenylalanine and the brain is irreversibly damaged unless a diet low in Phe is given in the first few weeks of life. Tyrosine is the precursor of some hormones (like the thyroid hormones) and the brown pigment melanin formed in hair, eyes and tanned skin. It reduces the requirement of Phe. Permanent deficiency of the enzyme-hypertyrosinaemia, a rear inborn error of metabolismcan cause liver and kidney failure unless treated with a synthetic diet low in Phe and Tyr (Bingham, 1977). Valine, an EAA is restricted in the treatment of maple syrup urine disease. When the present results were compared to amino acids profile of the eyes and brain of the African Giant pouch rat, it was observed that the following AA were all (in all the samples) more concentrated in the red viscera than in the eyes and brain (Oyarekua and Adeyeye, 2011) (g/100 g cp): Lys, Arg, Asp, Ser, Glu, Pro, Gly, Ala, Cys, Leu, Phe, whereas, His, Thr, Met, Val, Ile and Tyr had very close relationships.

Table 3 presents parameters on the quality of the protein of the samples. The EAA ranged between 40.2-43.8 g/ 100 g cp with a variation of 3.99%. These values were more than half the average of 56.6 g/100 g cp of the egg reference protein (Paul *et al*., 1976). The total sulphur AA (TSAA) of the samples were 3.26-3.60 g/ 100 g cp and these values were close to the value of 5.8 g/100 g cp recommended for infants (FAO/ WHO/ UNU, 1985). The aromatic AA (ArAA) range suggested for infant protein (6.8-11.8 g/100 g cp) (FAO/ WHO/ UNU, 1985) was very favourably comparable with the present report of 7.28-8.45 g/100g cp showing that the samples protein could be used to supplement cereal flours (Adeyeye, 2008a). The percentage ratio of EAA to the total AA (TAA) in the samples ranged between 46.4-48.4%. These values were well above the 39% considered adequate for ideal protein food for infants, 26 % for children and 11% for adults (FAO/WHO/UNU, 1985). The percentage of EAA/TAA for the samples could be favourably compared with other animal protein sources: 46.2% in *Zonocerus variegatus* (Adeyeye, 2005a), 43.7% in *Macrotermes bellicosus* (Adeyeye, 2005b), 54.8% in *Gymnarchus niloticus* (Trunk fish) (Adeyeye and Adamu, 2005) and 48.1-49.9% in brain and eyes of African giant pouch rat (Oyarekua and Adeyeye, 2011), whereas, it is 50% for egg (FAO/WHO, 1990). The TEAA in these results were close to the value of 44.4 g/100 g cp in soybean (Kuri *et al*., 1991), melon and gourd oilseeds with respective values of

Table 3. EAA, non-EAA, acidic, neutral, sulphur and aromatic acid contents of the fancy meats of African giant pouch rat (dry weight)

Amino acid	Tongue	Liver	Kidney	Heart	CV%
	(g/100 g crude protein)				
Total amino acid (TAA)	86.6	91.4	87.1	83.4	3.77
Total non-essential amino acid (TNEAA)	46.4	47.7	45.9	43.0	4.34
Total EAA - with His	40.2	43.8	41.3	40.4	3.99
- no His	37.9	41.5	38.2	37.4	4.81
% TNEAA	53.6	52.2	52.7	51.6	1.61
% Total EAA - with His	46.4	47.9	47.4	48.4	1.80
- no His	45.0	46.6	45.5	46.5	1.70
Total neutral amino acid (TNAA)	44.8	53.1	46.1	44.3	8.68
% TNAA	51.7	58.1	52.9	53.1	5.25
Total acidic amino acid (TAAA)	25.7	22.7	24.7	23.5	5.47
% TAAA	29.7	24.8	28.4	28.2	7.53
Total basic amino acid (TBAA)	16.1	15.6	16.3	15.6	2.24
% TBAA	18.6	17.1	18.7	18.7	4.29
Total sulphur amino acid (TSAA)	3.26	3.36	3.60	3.60	4.99
% TSAA	3.76	3.68	4.13	4.32	7.64
% Lys in TSAA	27.0	35.7	29.2	33.3	12.5
Total aromatic amino acid (TArAA)	8.45	7.95	7.28	7.68	6.2
P-PER	2.19	2.62	2.15	2.32	9.17
Leu/Ile ratio	1.67	1.82	1.78	1.90	5.34
Leu-Ile (difference)	2.68	3.45	2.85	3.29	11.8
% Leu-Ile (difference)	40.0	45.1	43.8	47.4	7.03
EAAI	1.l4	1.31	1.23	1.20	5.80
Isoelectric point (pI)	4.97	5.22	5.02	4.82	3.30

53.4 g/100 g cp and 53.6 g/100 g cp (Olaofe *et al*., 1994). The percentage of total neutral AA (TNAA) ranged from 51.7-58.1, indicating that these formed the bulk of the AA; total acidic AA (TAAA) ranged from 24.8-29.7 which were much lower than % TNAA, while the percentage range in total basic AA (TBAA) was 17.1-18.7 which made them the third largest group among the samples. The predicted protein efficiency ratio (P-PER) was 2.15-2.62. These results were highly comparable to the following literature values: 2.27 (skin) and 1.93 (muscle) of turkey hen (Adeyeye and Ayejuyo, 2007); 1.58 (brain) and 2.08 (eyes) of African giant pouch rat (Oyarekua and Adeyeye, 2011); 2.22 (*Clarias anguillaris*), 1.92 (*Oreochromis niloticus*) and 1.89 (*Cynoglossus senegalensis*) (Adeyeye, 2009a) but lower than in the values from various parts of fresh water female crab: 3.4 (whole body), 3.1 (flesh), 2.6 exoskeleton (Adeyeye, 2008b); fresh water male crab: 2.9 (whole body), 2.8 (flesh), 2.4 (exoskeleton) (Adeyeye and Kenni, 2008); 4.06 (corn ogi) and reference casein with PER of 2.50 (Oyarekua and Eleyinmi, 2004); 2.56

(cattle brain), 3.04 (pig brain), 2.68 (sheep brain), 3.26 (pig heart), 3.24 (pig kidney), 3.22 (pig liver), 3.00 (sheep heart), 2.57 (sheep kidney), 2.88 (sheep liver), 2.45 (sheep tongue) but better than 1.15 (cattle heart), 0.99 (cattle kidney), 1.20 (cattle liver), and 1.14 (cattle tongue) (Fornias, 1996). Other literature values were 1.21 (cowpea), 1.82 (pigeon pea) (Salunkhe and Kadam, 1989); 1.62 (millet ogi) and 0.27 (sorghum ogi) (Oyarekua and Eleyinmi, 2004); greater than 0.00 (raw sorghum), 0.23 (steeped sorghum) and 0.29 (malted sorghum) (Adeyeye, 2008a).

The Leu/Ile ratio was low in the samples (1.67-1.82) with CV% of 5.34; hence no concentration antagonism might be experienced in the giant pouch rat red viscera when used as protein source in food. The essential AA index (EAAI) ranged from 1.14-1.30. EAAI is useful as a rapid tool to evaluate food formulations for protein quality, although it does not account for difference in protein quality due to various processing methods or certain chemical reactions (Nielsen, 2002). The EAAI of defatted soybean is 1.26 (Nielsen, 2002); 1.10 (brain) and -1.10 (eyes) of African giant pouch rat (Oyarekua and Adeyeye, 2011), the present EAAI values were much better than the cited literature values. In the results of the isoelectric points (pI), there were various values (4.82-5.22). This type of observation had been made in African Giant pouch rat (Oyarekua and Adeyeye, 2011) in brain (4.28) and eyes (4.25); also in turkey meat: skin (4.41) and muscle (5.01) (Adeyeye and Ayejuyo, 2007). The calculation of pI from AA would assist in the quick production of the protein isolate of an organic product without going through the protein solubility determination to get the pI. Most animal proteins are low in Cys, for example: 36.3% in *M. bellicosus* (Adeyeye, 2005b), 25.6% in *Z. variegatus* (Adeyeye, 2005a); 35.5% in *Archatina marginata,* 38.8% in *Archatina archatina* and 21.0% in *Limicolaria* sp., respectively (Adeyeye and Afolabi, 2004); 27.3% - 32.8% in female fresh water crab body parts (Adeyeye, 2008b); 23.8-30.1% in three different Nigerian fishes (Adeyeye, 2009a); 13.3%-15.9% in male fresh water crab body parts (Adeyeye and Kenni, 2008); 26.0-26.5 % in turkey hen meat (Adeyeye and Ayejuyo, 2007); 20.8-28.2% in skin and muscle of African giant pouch rat (Oyarekua and Adeyeye, 2011) in their (Cys/TSAA) % values. The present results corroborated these literature observations with values of 27.0-35.7%. This type of results had also been observed in guinea fowl egg (14.0%) (Adeyeye, 2010); 44% in domestic fowl (Adeyeye, 2010); 26.2% (muscle) and 30.2 (skin) in guinea fowl (Adeyeye, 2011). In contrast, many vegetable proteins contain substantially more Cys than Met, examples (Cys/TSAA)%: 62.9% in coconut endosperm (Adeyeye, 2004) and in *Anacardium occidentale* it is 50.5% (Adeyeye *et al*., 2007); 58.9-72.0% (raw, steeped, germinated sorghum) (Adeyeye, 2008a); 51.2-53.1% (raw, steeped, germinated millet) (Adeyeye, 2009b). Thus, for animal protein diets or mixed diets containing animal protein, Cys is unlikely to contribute up to 50% of the TSAA (FAO/WHO, 1991). The percentage of Cys in TSAA had been set at 50% in rat, chick and pig diets (FAO/WHO, 1991). Cys can spare with Met in improving protein quality and also has positive effects on mineral absorption, particularly zinc (Mendoza, 2002; Sandstrom *et al*., 1989).

Table 4 shows the AA scores (AAS) of the samples based on whole hen's egg profile (Paul *et al*., 1976). The scores had values greater than 1.0 in Lys, Arg, Glu, and Gly (tongue); Lys, Arg, Glu, Pro and Gly (liver); Lys, His, Arg, Glu and Gly (kidney) and His, Arg, Glu and Gly (heart). Only Lys, Arg, Glu and Gly were greater than 1.0 in all the samples. Glycine had the highest score (1.40-2.67) in all the samples; the least score was Ser across the samples (0.38-0.50). The AAS

Table 4. Amino acid scores of the fancy meats of the African giant pouch rat based on whole hen's egg amino acid profile

Amino acid	Tongue	Liver	Kidney	Heart	CV %
Lys	1.09	1.12	1.03	0.97	6.32
His	0.97	0.96	1.30	1.26	16.3
Arg	1.15	1.05	1.12	1.0	4.17
Asp	0.99	0.91	0.98	0.99	3.99
Thr	0.51	0.82	0.77	0.52	24.9
Ser	0.50	0.47	0.45	0.38	11.3
Glu	1.26	1.09	1.19	1.08	7.43
Pro	0.76	1.11	0.84	0.92	16.5
Gly	1.62	2.67	2.07	1.40	28.9
Ala	0.83	0.77	0.73	0.75	5.61
Cy	0.49	0.67	0.58	0.67	14.3
Val	0.49	0.75	0.56	0.67	18.7
Met	0.74	0.68	0.80	0.75	6.63
Ile	0.72	0.75	0.65	0.65	7.30
Leu	0.81	0.92	0.78	0.84	7.19
Tyr	0.91	0.91	0.79	0.87	6.50
Phe	0.94	0.84	0.81	0.82	7.00

values in these results followed the pattern observed in the African Giant pouch rat skin and muscle (Oyarekua and Adeyeye, 2011). The red viscera of the African Giant pouch rat generally showed good comparisons with AA profile of the whole hen's egg. The CV% of the AAS ranged between 3.99-28.9. Table 5 contains the essential AA scores (EAAS) based on provisional amino acid scoring pattern (FAO/WHO, 1973). The EAAS greater than 1.0 in the tongue were Lys , Ile, Phe +Tyr and total; in liver they were Lys, Thr, Val, Ile, Leu, Phe +Tyr and total; for kidney Lys, Met +Cys, Phe +Tyr and total; for heart Lys, Met +Cys, Val, Phe +Tyr and total. Lys, Phe +Tyr and total were consistently greater than 1.0 in all the samples. The limiting AA (LAA) in the tongue was Thr (0.65), it was Met +Cys (0.95) in liver, it was Val (0.84) in kidney and Thr (0.66) in the heart. Although these would have been described as the LAA, however, the EAA most often acting in a limiting capacity are Met (and Cys), Lys, Thr and Try (FAO/WHO/UNU, 1985). Since Try was not determined, Thr would be limiting in tongue, liver and heart, whilst Met +Cys would be limiting in the liver. To make corrections for the LAA in the samples if they serve as sole sources of protein food therefore, it would be 100/65 (or 1.54) × protein of tongue, 100/95 (or 1.05) × protein of liver, 100/99 (or 1.01) × protein of kidney and 100/66 (or 1.52) × protein of heart (Bingham, 1977). The highest EAAS in the tongue was Phe +Tyr (1.41), it was Phe +Tyr (1.33) in the liver, it was Phe +Tyr in the kidney (1.21) and it was Phe +Tyr (1.28) again in the heart. The Table 6 shows the EAAS based on suggested requirement of the EAA of a preschool child (FAO/WHO/UNU, 1985). In the liver all the EAAS were greater than 1.0 whereas only Thr was less than 1.0 in the tongue (0.76) and the heart (0.78); hence Thr would be the LAA in both (tongue and heart), whereas, no LAA in the liver and Leu (0.98) would be the LAA in the kidney. Therefore, the corrections would be 100/76 (or 1.32) × protein of tongue, 100/78 (or 1.28) × protein of the heart and 100/98 (or 1.02) × protein of the kidney. The following values would show the position of the quality of the African giant pouch rat red viscera protein: the EAA requirements across board are (values with His) (g/100 g protein): infant (46.0), preschool (2-5 years) (33.9), school child (10-12 years) (24.1) and adult (12.7) and without His: infant (43.4), pre-school (32.0), school child (22.2) and adult (11.1) (FAO/WHO/UNU, 1985); from the present results based on these standards, we have: 37.4 g protein (with His) and 35.1 (no His) in tongue; 42.2 g protein (with His) and 39.9 (no His) in liver; 34.7 g protein (with His) and 31.6 (no His) in kidney; 36.0 g protein (with His) and 33.0 (no His) in heart. While, the present results would satisfy a high percentage of infant needs, they will satisfy the requirements of preschool children and above.

Table 5. Amino acid scores of the fancy meats of the African giant pouch rat based on the provisional amino acid scoring pattern

Amino acid	Tongue	Liver	Kidney	Heart	CV %
Lys	1.23	1.27	1.16	1.10	6.33
Thr	0.65	1.05	0.99	0.66	25.3
Met +Cys	0.93	0.95	1.03	1.03	5.34
Val	0.73	1.12	0.84	1.00	18.7
Ile	1.01	1.05	0.91	0.91	7.34
Leu	0.96	1.09	0.93	0.99	7.00
Phe +Tyr	1.41	1.33	1.21	1.28	6.44
Total	1.01	1.14	1.02	1.02	5.90

Table 6. Amino acid scores (g/100 g)of the fancy meats of the African giant pouch rat based on suggested requirement of the essential amino acid of a preschool child

Amino acid	Tongue	Liver	Kidney	Heart	CV %
Lys	1.16	1.20	1.10	1.04	6.22
His	1.22	1.21	1.65	1.59	16.6
Thr	0.76	1.24	1.16	0.78	25.4
Val	1.04	1.60	1.20	1.43	18.8
Met + Cys	1.30	1.34	1.44	1.44	5.16
Ile	1.44	1.50	1.30	1.30	7.30
Leu	1.02	1.16	0.98	1.05	7.33
Phe + Tyr	1.34	1.26	1.16	1.22	6.06
Total	1.15	1.29	1.18	1.18	5.14

Table 7 gives a brief summary of the AA profile in the four samples. Column under factor B means showed that the values were close with a range of 41.4 -45.8. However, the mean of factor A means and factor B means gave a value of 43.6 g/100 g as a total summary.

The chi square (X^2) test results were all low for all the parameters determined and the values were not significantly different at $\alpha = 0.05$ among the samples.

Table 7. Summary of the amino acid profiles into factors A and B

Amino acid composition (Factor B)	*Cricetomys gambianus* (Factor A)				Factor B means
	Tongue	Liver	Kidney	Heart	
Total essential amino acid	40.2	43.8	41.3	40.4	41.4
Total non-essential amino acid	46.4	47.7	45.9	43.0	45.8
Factor A means	43.3	45.8	43.6	41.7	43.6

Conclusion

This study has presented the amino acids data of the fancy meats (red viscera) of African Giant pouch rat (*Cricetomys gambianus*) female. It was found that the samples were good source of high quality protein of almost adequate or more than adequate essential amino acids, low Leu/Ile ratio and high protein efficiency ratio values thereby providing a probable premium quality meat. The samples were also very highly comparable to the red viscera of cattle, pig and sheep. The analytical results would also add information in the food composition of different meat.

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Enhancement of the Nutritional Value of Whey Drink by Supplementing with Leaves of *Moringa oleifera*

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Abstract: The effect of supplementing *Moringa oleifera* leaf powder (MOLP) on the nutritional and sensory characteristics of whey drink was investigated. Whey drink was supplemented with MOLP at four different concentrations i.e., 1% MOLP (T_1), 2% MOLP (T_2), 3% MOLP (T_3), 4% MOLP (T_4) and compared with a control (T_0). The addition of MOLP at any level did not have a negative effect on pH and acidity of whey drink. Iron content of T_4 increased from 0.17 to 115 mg/100 mL, total phenolic content of MOLP was 7.4 g/100 g on dry weight basis (gallic acid). Vitamin C increased from 1.46 to 2.20 mg/100 g in T_4. The overall acceptability score of T_4 was 6.9 out 9 (total score) which was more than 76%. These results suggest that nutritional value of whey can be increased by supplementing with 4% dry leaves of *M. oleifera* in the form of a whey based drink with acceptable sensory characteristics.

Keywords: *Moringa oleifera,* leaf powder, whey drink, iron, malnutrition

Introduction

Malnutrition results in retarded physical and mental growth as well as lack of resistance against diseases, whereas, protein deficiency results in poor health and reduced capacity for physical work (NNS, 2004). Plants have natural antioxidants and polyphenolic compounds that have anticarinogenic, cardiac and hepatic protective activity (Jeong *et al.,* 2004; Kris-Etherton *et al.,* 2002; Middleton *et al.,* 2000). *M. oleifera* is fast growing tree and its flowers, leaves and pods are consumed as vegetable and tender roots are preserved in the form of tasty pickles (Anwar *et al.,* 2007). It has high contents of protein and vitamins (Juliani *et al.,* 2009; Quarcoo, 2008; Fuglie, 1999).

Whey is the by-product of cheese, containing nearly half of the total solids present in milk (Walzem *et al.,* 2002). Cheese manufacturing units of Pakistan throw thousands of liters of untreated whey into the drain on daily basis which must be treated before discharge into the environment (Marwaha and Kennedy, 1998). Soft drinks may also be manufactured from whey by making use of different types of stabilizers and fruit concentrates of orange, apple, banana, mango and lemon (Niketic and Marinkovic, 1984). Keeping in view the utilization of whey, the present investigation was aimed to prepare nutritious whey drink by exploiting the massive nutritional potential of *M. oleifera*.

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Materials and Methods

Materials. Dried *M. oleifera* leaves were ground in a laboratory scale grinder (Moulinex), sieved through mesh size 50, stored in amber colour glass bottles, sealed and stored at -40 °C till further usage in this study. All the chemicals used in this study were HPLC grade and obtained from Sigma Chemical Co. (St. Louis MO).

Treatments. *Moringa oleifera* leaf powder (MOLP) was incorporated into whey drink at four different concentrations i.e., 1% MOLP (T_1), 2% MOLP (T_2), 3% MOLP (T_3), 4% MOLP (T_4), and compared with a control (T_0). The formulation of control comprised of banana pulp 5%, sugar 5%, citric acid (20% solution) 0.5%, carageenan 0.25% and whey to make volume up to the mark, colour and flavour 1 mL/L. All the ingredients were dissolved in the whey, pasteurised in the beakers at 65 °C for 30 min, immediately cooled down, filled in clean sanitised pet bottles and stored at 4 °C for further analysis. Each treatment was replicated three times.

Analysis. Fat, pH, acidity, protein, lactose, ash content and total solids were determined by following the respective methods AOAC 7.093, AOAC 7.094 and AOAC 7.095 (AOAC, 2000). Ca, K and Mg were analysed on atomic absorption spectrophotometer (Model: AA240, Varian, Australia) briefly, 5 g sample was taken into a conical flask, 10 mL of concentrated

nitric acid was added and contents of the flask were heated for 20 min. After cooling, 5 mL perchloric acid was added and again heated vigorously till the volume was reduced to 2-3 mL followed by cooling. The contents were then diluted in 50 mL volumetric flask by using distilled water. Absorbance of each mineral was determined on the respective wave length, also the unknown concentration determined by constructing calibration curve using 8 standards of each mineral (R^2= 0.9891 to 0.9961). Concentration of iron was determined by using the standard method AOAC 999.11 of AOAC (2000). Vitamin assay was performed on HPLC by following the respective methods AOAC 971.30, AOAC 948.26, and AOAC 975.42 (AOAC, 2000). Free fatty acids and peroxide values were determined by using standard methods cd 8-53 and cd 1-25 of Firestone (1997). For the determination of total phenolic contents of MOLP, 1 mL (methanolic extract of leaf powder) was poured into 11 mL screw capped test tube, 2 mL (0.2-molar) folin ciocalteu, 2 mL sodium carbonate (7.5%) added, mixed and stored in dark for 20 min at ambient temperature and absorbance measured at 765-nm in visible region on a double beam spectrophotometer (Shidmadzu, Japan). The concentration of phenolic compounds was measured on a standard curve constructed by using gallic acid as standard (R^2= 0.9943) by following the method of Anwar *et al.* (2007). The sensory evaluation of whey drink was conducted by a panel of 10 trained judges performed on a 9 point hedonic scale (1- the worst; 9- the best) as prescribed by Larmond (1987). The samples were evaluated for texture, taste, smell and overall acceptability. Samples were presented in glasses, coded with three digit random numbers and all servings were completely randomized. The data of triplicate experiments designed completely randomized (CRD). All data expressed as Mean±SD, the significant difference among the treatments calculated by using Duncan's Multiple Range Test SAS 9.1 Statistical Software.

Results and Discussion

Composition of whey drink supplemented with *M. oleifera* leaves. The results of chemical composition of whey drink show that fat, protein, lactose, ash and total solids content were 0.42%, 0.65%, 4.38%, 0.58% and 6.11%. Addition of MOLP at all the four levels did not have any negative effect on pH and acidity of supplemented whey drink (Table 1).

As the concentration of MOLP increased to 2%, fat content increased significantly ($P<0.05$). The highest fat content 0.64% observed in T_4 which was 52% over the control. Protein content of the supplemented whey drinks increased in a concentration dependent manner. Increase in protein content was 164% in T_4 as compared to the control. The increase in fat and protein content was due to the presence of higher concentration of these constituents in MOLP. Addition of MOLP at all levels increased the ash content of whey drinks significantly ($P<0.05$), T_1 and T_2 comparison with each other ($P>0.05$), increased to 52 and 65% in T_3 and T_4, respectively, as compared to the control. Increasing trend was observed in total solids with increased level of supplementation. The highest total solids recorded in T_4 (16.93%) followed by T_3 (15.84%) and T_2 (14.89%).

Total phenolic contents: Total phenolic content of MOLP was 7.4 g/100 g on dry matter basis (GAE). The total phenolic contents of *Sesamum indicum* cake extract was 1.94% on dry matter basis (Mohdaly *et al.,* 2011). In *M. olifera* concerentration of phenolic compounds was considerably higher than *S. indicum* (Mohdaly *et al.,* 2011) and canola hull (Naczk and Shahidi, 1998).

Minerals content of whey drink supplemented with *M. oleifera* leaves. Calcium content of all the treatments

Table 1. Effect of MOLP supplementation on composition of whey drink

Treatments	pH	Acidity	Fat	Protein	Lactose	Ash	TS
				(%)			
~~T_0~~	~~6.43±0.99^a~~	~~0.23±0.10^a~~	~~0.42±0.08^c~~	~~0.67±0.13^e~~	~~4.65±0.21^a~~	~~0.60±0.14^c~~	~~12.75±0.21^e~~
T_1	6.37±0.83^a	0.24±0.06^a	0.45±0.16^c	0.94±0.11^d	4.56±0.15^a	0.71±0.22^b	13.80±0.18^d
T_2	6.38±0.66^a	0.24±0.09^a	0.51±0.14^b	1.19±0.34^c	4.39±0.11^b	0.85±0.12^b	14.89±0.35^c
T_3	6.35±0.77^a	0.25±0.14^a	0.55±0.11^b	1.46±0.29^b	4.31±0.08^b	0.91±0.18^a	15.84±0.42^b
T_4	6.38±1.02^a	0.24±0.12^a	0.64±0.19^a	1.73±0.43^a	4.25±0.14^b	0.99±0.24^a	16.93±0.29^a

Means of triplicate experiment; means with same letters in same column are statistically non-significat ($P>0.05$); TS = total solids; T_0 = control without any addition of MOLP**;** T_1 = 1% MOLP; T_2 2% MOLP; T_3 3% MOLP; T_4 4% MOLP

increased in a dose dependent manner. The results showed the increasing level of calcium content of fortified whey drink by 10%, 19%, 31% and 47%, respectively, as compared to the control, while the effect on potassium content of the drinks was non-significant (Table 2). Potassium content ranged from 1226 to 1245 mg/L among different treatments and control. The elevation in calcium content of the experimental samples was due to the higher concentration of calcium in MOLP. The addition of MOLP at 4% level increased the iron content of the whey drink by 675% over the control.

The addition of MOLP at 1% level (T_1) increased the iron content from 0.17 mg/L to 44 mg/L which was 257% higher than the control (Aney *et al.,* 2009) while, studying the nutritional value of *M. oleifera* reported that the calcium, magnesium and iron content of 100g dried MOLP was 2000 mg, 1328 mg and 28.2 mg, respectively. As recommendations of FAO, WHO 400 mg and 1200 mg calcium is required on daily basis for the children of 1-3 years age and nursing women. Half of the total calcium requirement for nursing women may be easily and economically fulfilled by consuming just two glasses of 250 mL of whey drink supplemented with 4% MOLP, almost 75% of the calcium requirements of the children can be met by 250 mL of the fortified drink. *M. oleifera* leaves demonstrated higher vitamin A than carrots, more calcium and potassium than milk and banana with superior amino acid profile resembling to egg proteins (Juliani *et al.,* 2009). Milk is not a complete food because of its lower iron and vitamin C contents, fortification of milk with iron is usually practiced in poor and malnourished countries. Most of the strategies involve the usage of sulphates of iron, which are unnatural and accelerate the oxidative breakdown (Fox and McSweeney, 2003). The iron requirement of 10 and 15 mg per day for children and nursing women can be efficiently fulfilled by 250 and 300 mL of supplemented (T_4) whey drink. Feeding *M. oleifera* supplemented diet significantly improved the health status of school going babies and pregnant women in Senegal who gave birth to healthy babies (Juliani *et al.,* 2009).

Feeding 10 g leaves to pregnant women may provide adequate concentration of essential micro nutrients to save them from becoming anemic, physically and mentally weak (Fahey, 2005). This could be the simple, economical and efficient way of preventing and correcting the anemic conditions of millions of children and women of the poor nations who cannot afford medication and expensive nutritional supplements.

Table 2. Effect of MOLP supplementation on mineral content of whey drink

Treatments	Calcium	Potassium	Magnesium	Iron
	(mg/100 g)			
T_0	268±0.93^{e}	1245±6.13^{a}	35±0.43^{b}	0.17±0.03^{e}
T_1	297±0.81^{d}	1231±2.48^{a}	38±1.24^{b}	44±0.09^{d}
T_2	320±0.54^{c}	1226±1.05^{a}	42±0.06^{b}	75±0.34^{c}
T_3	353±0.77^{b}	1238±0.78^{a}	45±0.31^{a}	99±0.52^{b}
T_4	394±1.06^{a}	1241±3.16^{a}	48±0.19^{a}	115±0.05^{a}

Means of triplicate experiment; means with same letters in a column are statistically non-significant; refer Table 1 for the detail of treatments.

Vitamins content of whey drink supplemented with *M. oleifera* leaves. The addition of MOLP at all levels significantly ($P<0.05$) increased the vitamin B_5, B_2, B_6 and vitamin C content (Table 3). The increase in vitamins B_5, B_2, B_6 and C was 158%, 372%, 193% and 50%, respectively, in T_4. The increase in vitamin content was due to the chemical composition of the MOLP which resulted in higher concentrations of these vitamins in the whey drinks. The addition of MOLP did not have any effect on free fatty acids and peroxide value of whey drink at all levels. The recommended daily allowance of vitamin B_2 is 0.8 and 1.8 mg for children

Table 3. Effect of MOLP supplementation on vitamin content and stability of whey drink

Treatments	Vitamin B_5	Vitamin B_2	Vitamin E	Vitamin C	FFA%	PV (meq/kg)
	(mg/L)					
~~T_0~~	~~3.94±0.03^{e}~~	~~2.14±0.16^{e}~~	~~2.85±0.05^{e}~~	~~1.46±0.21^{e}~~	~~0.10±0.01^{a}~~	~~0.23±0.03^{a}~~
T_1	5.59±0.14^{d}	4.16±0.24^{d}	3.91±0.18^{d}	1.69±0.19^{d}	0.10±0.01^{a}	0.23±0.01^{a}
T_2	7.12±0.11^{c}	6.06±0.42^{c}	5.02±0.45^{c}	1.83±0.41^{c}	0.11±0.03^{a}	0.25±0.05^{a}
T_3	8.68±0.31^{b}	9.03±0.34^{b}	6.15±0.29^{b}	2.01±0.12^{b}	0.11±0.01^{a}	0.26±0.02^{a}
T_4	10.20±0.43^{a}	10.12±0.66^{a}	8.36±0.45^{a}	2.20±0.67^{a}	0.11±0.02^{a}	0.26±0.09^{a}

Means of triplicate experiment; means with same letters in same column are statistically non-significant; refer Table-1 for the detail of treatments; FFA = free fatty acids; PV = peroxide value.

and nursing women, 300 mL of supplemented whey drink at 2% MOLP can meet 100% body's requirement with respect to this vitamin. 423 and 17 mg/100 g, vitamin B_2 and C were present in Ethiopian *Moringa stenopetala* (Price, 1985). Anwar *et al.* (2007) studied the effect of *M. oleifera* addition on oxidative stability of some vegetable oils and found that blend of soybean and sunflower containing 80% (MOO) has induction time from 1.12 to 5.99 h, 1.47 to 6.22 h, recorded the increase in oxidative stability 435% and 323%, respectively.

Sensory evaluation. The results of sensory evaluation (Table 4) indicated that supplementation of MOLP up to T_2 level did not have any negative effect on texture and taste score. Texture and taste score of T_1 and T_2 were at par with the control ($P>0.05$). With the increasing increments of MOLP, the score for these two parameters decreased. Some panelists criticised T_3 and T_4 for relatively higher coarse texture. Level of criticism for T_4 was not adverse as the people are already habitual of adding green leaves of coriander and other herbs in the butter milk. Quarcoo (2008) studied the development of *M. oleifera* based beverage and reported that addition of increasing concentration of *M. oleifera* leaf powder tended to make the colour greener and decreased the colour score. The low colour score was attributed to the uncommon green colour of the beverage; the lower concentrations were rated higher for this parameter. This problem could have been resolved by homogenising the supplemented whey drinks at high pressure. Homogenisation is commercially carried out to make the nectars and high fruit preparations homogenous and smooth (Spreer, 2005). Score for smell of all the treatments and control was non-significantly influenced from each other. The overall acceptability score of T_4 was 6.9 out of 9, which was more than 76% of the total score.

Table 4. Effect of MOLP supplementation on sensory characteristics of whey drink

Treatments	Texture	Taste	Smell	Overall acceptability
T_0	7.6 ± 0.11^a	7.9 ± 0.23^a	7.5 ± 0.08^a	7.5 ± 0.03^a
T_1	7.3 ± 0.08^a	7.6 ± 0.09^a	7.4 ± 0.02^a	7.2 ± 0.14^a
T_2	7.4 ± 0.14^a	7.5 ± 0.11^a	7.5 ± 0.04^a	7.3 ± 0.05^a
T_3	7.0 ± 0.03^b	7.0 ± 0.17^b	7.2 ± 0.19^a	7.0 ± 0.09^b
T_4	6.8 ± 0.06^b	6.6 ± 0.14^b	7.2 ± 0.12^a	6.9 ± 0.04^b

Means of triplicate experiment; means with same letters in same column are statistically non-significant; refer Table 1 for the detail of treatments.

Conclusion

The addition of *M. oleifera* leaf powder at all concentrations improved the nutritional value of the whey drink. Protein and iron content significantly increased in the supplemented drinks. 300 mL of supplemented whey drink can fulfill 100% requirement of vitamin B_2 of nursing women. Highly nutritious whey drink can be successfully prepared by the addition of MOLP up to 4% level; whey drink supplemented with MOLP should be homogenised for better sensory characteristics.

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Evaluation of the Functional Food Potential of Bamirad (a Ginger-Spiced) Cheese Produced in the Western Highlands of Cameroon

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Abstract. In this study, cheese was modified to enhance its functional characteristics thereby encouraging its consumption. Consequently, a ginger-spiced cheese (Bamirad) was produced and the effects of feed supplementation with cheese on blood lipid profile were evaluated using 36 male Wistar rats in four groups. Total cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol and triacylglycerols were determined. There were significant ($P<0.05$) changes indicating: weekly increases of triacylglycerols for all treatments, higher total cholesterol for the 0.0 % ginger treatment, and a decline of low-density lipoprotein for 1.0 % ginger treatment. The LDL/HDL ratio was very low, indicating that this cheese is a functional food, a potential exploitable for the well-being of the consumer.

Keywords: Bamirad cheese, functional food, lipid profile.

Introduction

Cheese is a food item obtained by the coagulation, draining and ageing of milk proteins. When consumed, like all other foods, provides energy, nutrients for growth and replacement or regulatory substances for proper physiological processes of the body. However, there is the growing phenomenon of functional foods attributed to food substances endowed with other benefits beyond basic nutritional requirements. Practically, a food substance is regarded as functional if it can beneficially affect the body beyond adequate nutrition, in a way which is relevant to either the state of well-being and health or the reduction of the risk of developing a disease (Roberfroid, 2000).

The functional foods phenomenon within the past decade, created a worldwide impact in the food industry, where activities were geared towards finding solutions to consumers' worries and preoccupations about their health and well-being. Indeed, cancers, diabetes, cardiovascular, inflammatory and other chronic diseases have been particularly deadly, being responsible for 60% of the causes of human deaths worldwide with cardiovascular diseases (CVDs) most prominent. In developing countries (Sub-Saharan Africa, North-East Asia, Latin America), the prevalence of CVDs is more than 20% being almost the same as that of infectious and nutritional diseases formerly classified as the most common in these regions (WHO, 2003).

Spices are essentially plant substances, which in addition to imparting desirable flavours to food, are endowed with therapeutic potentials. Essential oils, phenols and flavanoids involved in reducing the risk of vascular disease and heart attacks (Abdou-Bouba *et al*., 2010; Li *et al*., 2007). Furthermore, compounds that exhibited antimicrobial, anti-inflammatory, antioxidant and lipid lowering effects have also been identified in some spices including: ginger, onions, garlic and parsley (Milner, 2000).

Dairy products, though very good protein sources have been implicated in high blood cholesterol levels. Although cheese by its nature of being a fermented milk product is a functional food, the incorporation of spices (Kim *et al*., 2006; Karvonen *et al*., 2002; During, *et al.,* 2000) could re-enforce its functional food capacity. The Bamirad cheese, in which ginger was added during manufacture, was evaluated for its functional food potential. The study was carried out

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focussing mainly on the evaluation of the effects of Bamirad cheese on blood lipid profile of male rats in which the cheese was a supplement in the rats' feed.

Materials and Methods

Meterials. The cheese was made following the procedure described earlier (Mendi *et al.,* 2009). In the study, two levels (1.0 and 1.5 % ginger powder) of spiced-cheese were tested. The cheese was made in the food technology laboratory of the Institute of Agricultural Research for Development (IRAD) Bambui in the north-west region and transported in ice-packed flask to the veterinary research laboratory of IRAD Wakwa-Ngaoundere in the Adamaoua region and stored in a refrigerator till used.

Animal feed. Adult male Wistar rats were obtained from the animal house of the faculty of science of the university of Yaoundé I, Cameroon and transported in plastic cages to IRAD Wakwa-Ngaoundere.

Methods. After a wash out period of one week, 36 rats were randomly distributed into four groups (average weights ranged from 229.3 to 244.4 g) for a complete randomised block (animals' weights) design study. The ingredients used to compound the rats' chow were: wheat bran, corn meal, soya bean meal, fish meal and common salt.

The groups were randomly assigned to feed supplementation regimes as follows: animals of group A consumed plain cheese (0.0% ginger), those of group B received cheese spiced at1.0% ginger, and the group C animals ate cheese spiced at 1.5% ginger, while the group D animals did not receive cheese as supplement.

The 9 rats in each group were distributed into three polypropylene base standard rats' cages (3 rats/cage) and were housed in a well ventilated, natural day light/night darkness cycle, laboratory room, with water and the basic feed ad libitum. Cheese (30 g/cage) was served first every morning before the basic feed. Baseline data were collected prior to start of cheese supplementation.

Lipid profile. Weekly blood samples were collected after an overnight fasting, by caudal vein puncture in 1.5 mL Eppendorf tubes (Fisher Bioblock Scientific 2002- France) that contained ethylene diamine tetra-acetic acid (EDTA) as anti-coagulant. The blood was centrifuged (SIGMA 3K 20 Germany) at 5000 rpm (revolutions per minutes) for 15 min and then the plasma was obtained and used for the determination of triacylglycerols, total cholesterol, low-density lipoprotein (LDL) cholesterol.

On the last day of feeding, the rats were sacrificed by chloroform anaesthesia and blood was collected by cardiac puncture. There was enough plasma for the determination of high density lipoprotein cholesterol (HDL) in addition to the other lipid parameters that were being assayed.

Total cholesterol. The determination of total cholesterol was done by using cholesterol liquid kits, enzymatic colorimetric test. CHOD-PAP method for the "*in vitro*" determination of cholesterol in serum or plasma (QUIMICA CLINICA APLICADA S.A., Spain). In this method, total cholesterol was determined by oxidation, with concomitant release of hydrogen peroxide which was assayed.

HDL cholesterol. The determination of high-density lipoprotein cholesterol (HDL) was done using the human cholesterol liquicolor test kit, with a precipitant and a standard following the semi-micro procedure (HUMAN Gesellschaft für Biochemica und Diagnostica mbH-Germany).

The principle was that the chylomicrons, very low density lipoproteins VLDL and low density lipoproteins LDL are precipitated by addition of phosphotungstic acid and magnesium chloride. After centrifugation the supernatant fluid contains the HDL fraction, which is then assayed for HDL-cholesterol with the human cholesterol liquicolor test kit.

The reagents comprised the precipitant for semi-micro assay that was diluted with 20 mL of distilled water for the contents of one bottle and the standard solution which was used as supplied. The procedure commenced with precipitation that was done by pipetting 0.2 mL of sample (serum) into centrifuge tubes, 0.5 mL of precipitant added, contents were well mixed and allowed to stand for 10 min at room temperature (18-23 °C) before centrifugation for 5 min. The clear supernatant was separated from the precipitate and used for the determination of the cholesterol concentration.

Data analysis. The changes in variables were calculated as follows:

$$\frac{\text{value determined at week} - \text{initial value}}{\text{initial value}} \times 100 = \% \text{ change}$$

Data were subjected to the analysis of variance (ANOVA) using SigmaPlot software version 11.0 with

Holm-Sidak method to compare means at the 95% significant level.

Results and Discussion

Lipid profile of rats. The plasma levels of triacylglycerols, total cholesterol and LDL cholesterol of rats before the study are presented in Table 1, where, there were significant ($P<0.05$) differences. The figures that follow illustrate the differences observed after the feeding period.

Table 1. Lipid profile of rats before start of cheese supplementation

Lipid profile	Rat groups/feed regime (% ginger spice)			
(mmol/L)	A (0.0)	B (1.0)	C (1.5)	D (no cheese)
Triacylglycerols	$0.84^{a}\pm0.08$	$0.55^{b}\pm0.20$	$0.84^{a}\pm0.08$	$0.69^{ab}\pm0.23$
Total cholesterol	$0.60^{b}\pm0.12$	$0.95^{a}\pm0.22$	$0.82^{ab}\pm0.20$	$0.85^{ab}\pm0.21$
LDL cholesterol	$0.48^{a}\pm0.15$	$0.55^{a}\pm0.15$	$0.53^{a}\pm0.10$	$0.61^{a}\pm0.21$

Averages ± standard deviations (n=9); values with different letter superscript along the same row denote a significant difference at $P<0.05$.

Triacylglycerols. There were significant ($P<0.05$) weekly exponential increases for all treatments (Fig. 1).

Total cholesterol. The weekly increase of total cholesterol was significantly ($P<0.05$) higher for the 0.0 % ginger-spiced cheese treatment only (Fig. 2).

LDL cholesterol. The weekly decline of LDL cholesterol was significant ($P<0.05$) for the 1.0% ginger-spiced cheese treatment only (Fig. 3).

The effects of the different diets fed to rats in four experimental groups, on their plasma lipids revealed an increase of triacylglycerols (TAG) and total cholesterol (TC), but a decrease of LDL cholesterol for all the treatments. The weekly increases of TAG were significant ($P<0.05$) for all treatments, while, the changes of TC and LDL were significantly ($P<0.05$) different for the 0.0 % ginger-spiced cheese treatment only.

Dairy products like cheese and butter are known for their ability to raise blood total cholesterol levels (Lokuruka, 2007). The role played by phytochemicals in blood lipid modification when consumed in diets was evident in these observations, accounting for the low values of the triacylglycerols and total cholesterol increases for the ginger-spiced cheese diet groups. Similarly, the reduction of plasma and hepatic cholesterol levels had been reported in mice when fed ground whole flaxseed diet as compared to the control diet of 0.1% cholesterol and 30% kcal fat. Also, soya bean protein

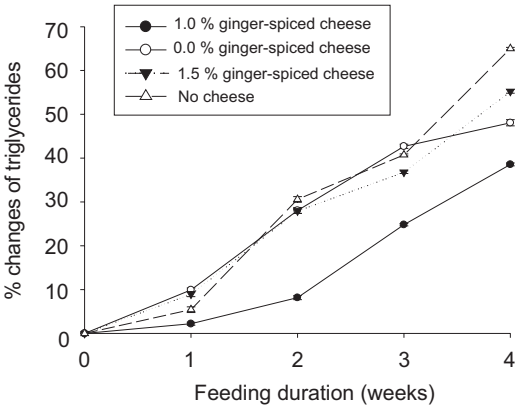

Fig.1. The effects of cheese supplementation on plasma triacylglycerols.

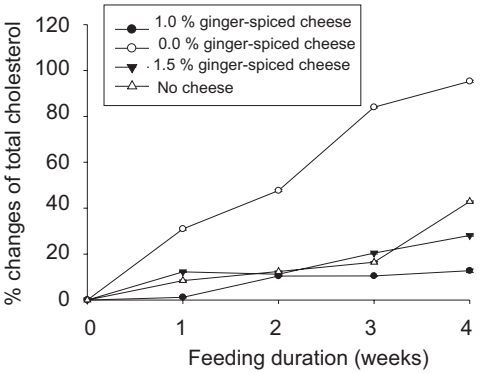

Fig. 2. Variation of total cholesterol of rats supplemented with cheese.

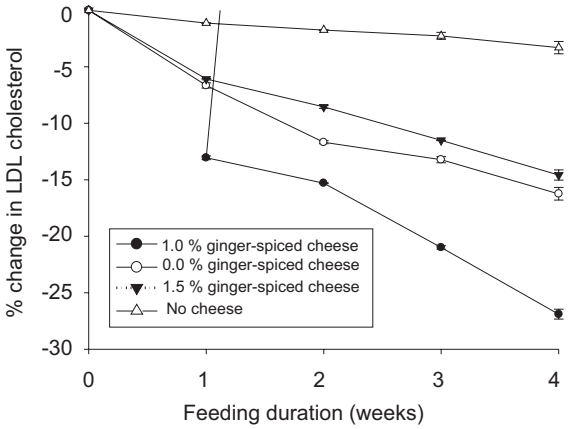

Fig.3. Variation of plasma LDL cholesterol of rats supplemented with cheese.

based diets have been observed to effectively lower plasma total cholesterol, triacylglycerols and LDL cholesterol compared to the control cholesterol-rich diet (Pellizzon *et al.*, 2007; Pittaway *et al*., 2007; Sheridan *et al.,* 2007; Yang *et al.*, 2007). In some studies, for example, Al-Amin *et al.* (2006) observed that aqueous extracts of ginger had actually been used to treat diabetic rats, lowering their serum triacyl-glycerols and cholesterol levels by 41 and 44%, respectively.

The overall reduction in LDL cholesterol, usually termed the "bad" cholesterol further demonstrated the important role of phytochemicals (Al-Numair, 2009) in blood lipids since the control diet was also rich in soya bean and corn oil, all of which are favourable in cholesterol management. However, the 1.0% ginger-spiced cheese demonstrated additional functional advantage of this food product.

Though cheese and other dairy products are rich in saturated fats and are often associated with the raising of blood cholesterols levels, the type and nature of fat, other nutrients in the diet and effect on lipid types differ. The prominent dairy saturated fatty acids, palmitic, myristic and lauric are directed to the formation of triacylglycerols immediately after absorption from the diet, but only the myristic and lauric acids raise plasma total cholesterol. Long chain fatty acids are said to have an adverse effect on LDL cholesterol (Lukuruka, 2007) so the decrease of LDL observed in the study was due in part to the cheese itself, and then the ginger effect.

The effects of the different diets on HDL. When the animals were sacrificed on the last day of the experiment, it was possible to have a good quantity of plasma for the determination of the high-density lipoprotein (HDL) cholesterol. Table 2 shows all lipid profile characteristics including HDL cholesterol which was significantly ($P<0.05$) lower for the 1.5% ginger-spiced cheese treatment. HDL is the "good" cholesterol as opposed to LDL the "bad" type. The LDL/HDL ratio was only slightly lower for the 0.0% ginger-spiced cheese treatment compared to the group not supplemented. When this ratio is low, the risk of developing associated diseases is also low (Ohlsson, 2010).

Table 2. The effects of cheese consumption on blood HDL cholesterol and other lipids of rats

Lipid profile (mmol/L)	Treatments (% ginger powder) A(0.0)	B(1.0)	C(1.5)	No cheese
Triacylglycerols	1.00^{bc}±0.08	0.79^{c}±0.17	1.27^{a}±0.2	1.13^{ab}±0.15
Total cholesterol	1.16^{b}±0.24	1.08^{a}±0.34	1.05^{a}±0.09	1.22^{a}±0.25
LDL cholesterol	0.40^{b}±0.06	0.41^{b}±0.09	0.45^{b}±0.15	0.59^{a}±0.09
HDL	0.21^{a}±0.14	0.16^{a}±0.09	0.09^{b}±0.04	0.15^{a}±0.10
LDL/HDL ratio	1.2±0.8	1.1±1.2	2.8±0.4	2.0±1.9

Averages ± standard deviations (n=9);values with different letter superscript along the row denote a significant difference at $P<0.05$.

Generally, fermented food products especially those of dairy origin, improve on the LDL/HDL ratio (Ajayi and Ajayi, 2009 Krissansen, 2007) hence the low values as observed.

The changes in the lipid profile characteristics of rats could also be associated with the number of beneficial microorganisms in their gut. The presence of fermentation bacteria in the gut had been responsible for an increase in propionate, hydrolysis of glycine and taurin-conjugated bile acids, enhancement of the excretion of bile acids in faeces, all of which end up in the lowering of blood cholesterol (Ohlsson, 2010 Adesokan *et al.*, 2009; Oozeer *et al*., 2002). Similarly, Safalaoh (2006) found that some microorganisms when included in poultry feed actually contribute to body weight gain of the birds.

Conclusion

In the evaluation of the functional food potentials of Bamirad cheese, it could be concluded that: the consumption of cheese in normal diets increases levels of triacylglycerols and total cholesterol in the blood, but the increase by ginger-spiced cheese diet is not as great as that of the plain cheese; ginger-spiced cheese lowers LDL cholesterol and improves on the LDL/HDL ratio hence, Bamirad cheese positively influences lipid metabolism.

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Prevalence and Diagnostic Test Comparison of Brucellosis in Cattle in Pabna and Mymensingh Districts of Bangladesh

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Abstract. Present study was undertaken to determine the seroprevalence of brucellosis in cattle of Pabna and Mymensingh districts in Bangladesh. A total of 260 cattle sera samples were collected from Pabna and Mymensingh districts. The epidemiological data were collected by structured questionnaire. RBT and SAT were used as screening tests and further confirmed by I-ELISA. The seroprevalence of *Brucella* in cattle was estimated to be 4.23%, 3.07% and 2.31% by RBT, SAT and I-ELISA, respectively. The comparison of the serological tests result revealed the highest prevalence in RBT than SAT and I-ELISA. The prevalence of *Brucella* was 2.5% in Pabna and 2.14% in Mymensingh. It was observed that, a higher prevalence of *Brucella* was found in female (2.67%) than in male (1.82%), natural breeding (2.67%) than artificial breeding (1.81%), in aged animals (3.33%) than young (1.25%). But these differences were not statistically significant. There exists significant difference between prevalence of *Brucella* in cattle with history of abortion than without history of abortion (P value=0.013).

Keywords: brucellosis, cattle, diagnosis, epidemiology, Bangladesh

Introduction

Brucellosis is a zoonotic disease caused by different species of the genus *Brucella* that are pathogenic for a wide variety of animals and humans. In animals, brucellosis mainly affects reproduction and fertility, reduces the survival of newborns and milk yield. Mortality in adult animals is insignificant (Sewell and Brocklesby, 1990). According to the Food and Agricultural Organization (FAO), the World Health Organization (WHO) and the World Organization of Animal Health (OIE), brucellosis is considered to be the most widespread zoonosis worldwide (Mustafa and Nicoletti, 1993).

Although it has been eradicated in many developed countries in Europe, Australia, Canada, Israel, Japan and New Zealand (Geering *et al*., 1995) in some other areas it has emerged as a major zoonotic disease in sheep and goats. The importance of brucellosis was primarily due to its public health significance and economic loss to the animal industry (WHO, 1971). Bangladesh has been reported as an endemic country for brucellosis because of a considerable number of human and animal populations are exposed to the infection each year.

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Brucellosis in humans is caused by exposure to livestock and livestock products. Infection can result from direct contact with infected animals and can also be transmitted to consumers through raw milk and milk products. Brucellosis spreads between animals in a herd and the disease is a systemic infection that can involve many organs and tissues. Once the acute period of the disease is over, symptoms of brucellosis are mostly not pathognomonic, however, the organism can be located in the supramammary lymph nodes and mammary glands of 80% of infected animals. Thus they continue to secrete *Brucella* in their body fluids (Redkar *et al*., 2001).

Under the name Malta fever, the disease (now called brucellosis) first came to the attention of British medical officers in the 1850s in Malta during the Crimean war. The causal relationship between the organism and disease was first established in 1887 by Dr. David Bruce (Wilkinson and Lise, 1993). Brucellosis is endemic worldwide including Bangladesh (Das *et al*., 2008) but often a neglected disease.

It causes a great economic loss to the livestock industries through abortion, infertility, birth of weak and dead offspring, increased calving interval and reduction of milk yield (Rahman *et al.*, 2006).

In Bangladesh it was first reported in cattle in 1967 (Mia and Islam, 1967), and in humans in 1983 (Rahman *et al*., 1983). Islam *et al*. (1983) estimated the annual economic loss in Bangladesh due to bovine brucellosis in indigenous cows as 720,000 EUR (total) and 12000 EUR per 1000 cross-bred cows and a total of 276000000 EUR in cross-bred cows. Recently brucellosis has been reported in cattle, buffalo, sheep, goat and pig in different regions of Bangladesh (Rahman *et al*., 2011a, 2011b; Rahman *et al*., 2010; Nahar and Ahmed, 2009; Uddin *et al*., 2007a, 2007b; Rahman *et al*., 2006; Amin *et al*., 2004). The diagnosis of brucellosis is confirmed by isolation of *Brucella* spp. by bacteriological culture or by the detection of an immune response by serological test to its antigens (Orduña *et al*., 2000). But the diagnosis of brucellosis based exclusively on *Brucella* isolation presents several drawbacks. The slow growth of *Brucella* may delay diagnosis for more than 7 days and also, the sensitivity is often low, ranging from 50 to 90% depending on disease stage, *Brucella* spp., culture medium, quantity of bacteria and culture technique employed (Gotuzzo *et al*., 1986). Hence, the serological tests are important for diagnosis of brucellosis.

Serological test like the rose Bengal test (RBT), slow agglutination test (SAT), mercaptoethanol test, enzyme linked immunosorbent assay (ELISA) and complement fixation test (Islam *et al*., 1983) are generally used for the detection of *Brucella* infection in animals. Enzyme linked immunosorbent assay (ELISA) has been evaluated for many years for the detection of serum antibody to *Brucella* in domestic animals. It has gained popularity over recent years as an alternative to other serological tests because it has several advantages compared with other tests such as, (a) it is direct method of identification of specific antibody, (b) it is more sensitive test than the slow aggluti-nation test, (c) the antibody enzyme conjugate employed has light chain reactivity and thus able to detect all classes of antibody. Despite having these advantages, there has been limited use of ELISA for the diagnosis of brucellosis in Bangladesh. Therefore, the present study was designed to diagnose brucellosis by adopting I-ELISA as well as RBT and SAT to detect antibodies to *Brucella* organism and to identify the risk factor and distribution of brucellosis in cattle in Pabna and Mymensingh districts of Bangladesh for prevention and control.

Materials and Methods

A total of 260 blood sera samples were collected from cattle of Pabna and Mymensingh districts of Bangladesh. Among cattle sera samples, 120 were collected from Bhangoora, Shordarpara, Kashipur and Gojatola of Pabna and 140 samples were collected from BAU Veterinary Clinic area, Sasmore, Sutiakhali and Digharkanda of Mymensingh during the period from May to December, 2011 (Table 1). Sampling was carried out as multistage sampling with the farm being selected first in the study area and then cattle randomly selected within each farm. The sampling frame within each farm was the list of all cattle on farm record. The sampling unit consisted of the animals selected from the list of all cattle within each farm using computer generated random numbers. The questionnaire based data on age, sex, breeding strategy, pregnancy status, area, history of abortion in cows were recorded.

Table 1. Collection of serum samples from cattle in Pabna and Mymensingh districts

Area/location	No. of cattle samples
Pabna district	120
Mymensingh district	140
Total	260

Blood and sera samples collection. Animals were restrained with the help of the owner. Then the site of blood collection at the jugular furrow was soaked with iodine or alcohol. About 5-7 mL of blood was collected from the jugular vein of each cattle using a sterile disposable syringe and needle and was kept undisturbed on a tray for at least 1 h at room temperature in a slightly inclined position to facilitate clotting and separation of serum. After this period, the clotted blood samples with sera were transferred to a refrigerator and were kept overnight at 4 °C. Then the blood samples with sera were centrifuged at 3000 rpm for 10 min. Later on, the sera were aliquated into sterilised labeled Eppendorf tube and stored at -20 °C until used.

Serological study. The serological test for the diagnosis of brucellosis in cattle was performed by rose Bengal test (RBT), slow agglutination test (SAT) for screening and indirect enzyme linked immunosorbent assay (I-ELISA) for confirmatory diagnosis.

Rose Bengal plate test. Rose Bengal test (RBT) was performed according to the procedure as described by OIE (2004) which is being routinely used and described previously by Uddin *et al*. (2007a, 2007b) using *B. abortus* antigen (obtained from Dae Sung Microbiological Lab, South Korea). The serum samples and *B. abortus* antigen were kept 1 h at room temperature before starting the test. Thirty µL of each serum to be tested was placed on a glass plate circled approximately 2 cm in diameter. Then the vial of antigens was shacked gently and 30 µL of antigen was put beside each of the sera. The antigens and the serum were mixed on the plate with a stirrer and spread over the entire area enclosed by the circle. Then the plate was placed on a mechanical rotator as 80-100 rpm for 4 min and the reading was noted immediately. Any agglutination or precipitation was considered as positive, whereas, no reaction (negative) indicated the absence of *Brucella* antigen in the sera.

Slow agglutination test (SAT). SAT was carried out with EDTA as described by Garin *et al*. (1985). The SAW (synbiotics, concentrated suspension of *B. abortus*, Weybridge, stain 99) antigen was diluted (1 mL antigen with 19 mL SAT buffer solution). The SAT buffer was prepared by adding 0.93 g EDTA (5 mM, Triplex®) to 500 mL PBS, where PBS was prepared by adding 5 tablets of PBS (Dulbecco-A, Oxoid, UK) to 500 mL distilled water. Briefly, the slow agglutination test was performed in flat bottom 96 well micro plates. At first for each test serum, a row of 3 wells of the 96 well micro plates was selected to make double dilution of the sera. 168 µL of SAW buffer was pipetted in first well and 100 µL in the 2nd well and 3rd well, respectively, of the micro plate. Then 32 µL of serum was added in 1st well (dilution 1/6.25) after well mixing of the serum and PBS EDTA in the 1st well and 100 µL was taken from this well and was placed in the second well (1/12.5). 100 µL from the 2nd well was transferred into the 3rd well and finally 100 µL of liquid in excess was discarded from 3rd well. Note that, all wells contained 100 µL. Then in each well 100 µL of standardized SAW antigen was added. This gives the serial serum dilution of 1/12.5, 1/25, 1/50. The plate was then incubated at 37 °C for 24 h (+/-4 h) for reading. After 24 h, the agglutination reaction was observed by using a magnifying mirror against illumination source. Notably, for every group of samples tested, a positive control serum was included. Reading was taken on the basis of this protocol and the standardization was performed (75% agglutination of the OIEISS). The results were interpreted according to instruction of Veterinary Agrochemical Research Centre (Groeseleaberg 99, 1180 Brussels, Belgium).

Indirect enzyme linked immunosorbent assay (I-ELISA). All the samples found to be positive in RBT were further confirmed using I-ELISA. The assay was performed according to the protocol provided by the manufacturer's instructions (Svanova Biotech AB, art No.10-2700-10, SE-751 83 Uppsala, Sweden).

All reagents supplied by the manufacturer company were equilibrated to room temperature 18 to 25 °C (64 to 77 °F) before use. An amount of 100 µL of sample dilution buffer was added to each well that would be used for serum samples and serum controls. After that 4 µL of positive control serum (reagent A) and 4 µL of negative control serum (reagent B) was added, respectively, to selected wells coated with *B. abortus* antigen. For confirmation purposes it was run the control sera in duplicates. The plate was shaken thoroughly and sealed then incubated at 37 °C (98.6 °F) for l h. The plate was rinsed 3 times with PBS-Tween buffer and filled up the wells at each rinse, emptied the plate and tapped thoroughly to remove all remains of the fluid. Then 100 µL of HRP conjugate was added to each well and incubated at 37 °C (98.6 °F) for 1 h. The plate was rinsed again according to the previous way. Then 100 µL substrate solution was added to each well and incubated for 10 min at room temperature 18 to 25 °C (64 to 77 °F). Begin timing after the first well was filled. The reaction was stopped by adding 50 µL of stop solution to each well and mixed thoroughly. The stop solution was added in the same order as the substrate solution was added. The optical density (OD) of the controls and samples was measured at 450 nm in a micro plate photometer. The OD was measured within 15 min after the addition of stop solution to prevent fluctuation in OD values. Any change in colour observed by naked eye indicated positive reaction.

Statistical analysis. The questionnaire-based data was processed in Microsoft Excel and analysed in SPSS. The z-test for proportions was used to compare the results between the serum tests. The z-test for proportions was done to find out the significant differences in the prevalence of *Brucella* based on the result of RBT in terms of age, sex, history of abortion, breeding strategies and study area.

Results and Discussion

A total of 260 sera samples from cattle were collected from Pabna and Mymensingh, 120 were collected from cattle of Pabna district and 140 from cattle of Mymensingh district (Table 1). The sera were tested by rose Bengal test (RBT), slow agglutination test (SAT) and indirect enzyme linked immunosorbent assay (I-ELISA) and the results are shown in Table 2 and 3. The overall prevalence of *Brucella* was found to be 4.23%, 3.07% and 2.31% by RBT, SAT and I-ELISA, respectively. It was shown that out of 260 cattle sera examined by RBT, 11 cattle sera showed positive reaction to RBT with a prevalence of 4.23%; 8 were positive to SAT with a prevalence of 3.07% and 6 to indirect ELISA with the prevalence of 2.31% (Table 2). The comparison of the serological test result revealed the highest prevalence in RBT than SAT and I-ELISA. But the prevalence of *Brucella* determined by RBT, SAT and I-ELISA did not differ significantly at 5% level of significance (P value=0.456).

Out of 260 cattle, 110 were male and 150 were female. The prevalence of *Brucella* in female was 4.67%, 3.33% and 2.67% in RBT, SAT and I-ELISA, respectively, and in male 3.64%, 2.73% and 1.82% in RBT, SAT and

Table 2. Overall seroprevalence of brucellosis in cattle based on RBT, SAT and I-ELISA

Total no. of samples collected and tested	No. of positive reactors			Percentage of positive reactors			Level of significance
	by RBT	by SAT	by I-ELISA	by RBT	by SAT	by I-ELISA	
260	11	8	6	4.23%	3.07%	2.31%	NS

NS = not significant at 5% level of significance (P value=0.456).

Table 3. Demographic factors related seroprevalence of brucellosis in cattle based on RBT, SAT and I-ELISA

No. of sera samples collected and tested		No. and % of positive reactors						#Level of significance
		by RBT	95% CI	by SAT	95% CI	by I-ELISA	95% CI	
Age								
< 2 years	60	2(3.33%)	1.21-7.87%	1(1.67%)	1.57-4.91%	1(1.67%)	1.57-4.91%	NS
≥ 2-4 years	80	3(3.75%)	0.41-7.91%	2(2.5%)	0.92-5.92%	1(1.25%)	1.18-3.68%	
> 4 years.	120	6 (5.0%)	1.1-8.9%	5(4.17%)	0.59-7.75%	4(3.33%)	0.12-6.54%	
Sex								
Male	110	4(3.64%)	0.14-7.14%	3(2.73%)	0.32-5.78%	2(1.82%)	0.68-4.32%	NS
Female	150	7(4.67%)	1.29-8.05%	5(3.33%)	0.46-6.2%	4(2.67%)	0.09-5.25%	
History of abortion								
Yes	20	3(15%)	0.65-30.65%	2(10%)	3.15-23.15%	2(10%)	3.15-23.15%	*
No	240	8(3.33%)	1.06-5.6%	6(2.25%)	0.37-4.13%	4(1.67%)	0.05-3.29%	
Breeding								
Breed by AI (Cross breed)	110	4(3.64%)	0.14-7.14%	3(2.73%)	0.32-5.78%	2(1.81%)	0.68-4.31%	NS
Natural breeding (Indigenous)	150	7(4.67%)	1.29-8.05%	5(3.33%)	0.48-6.2%	4(2.67%)	0.1-5.25%	
Location/Area								
Pabna	120	6(5%)	1.1-8.9%	4(3.33%)	0.12-6.54%	3(2.5%)	0.29-5.29%	NS
Mymensingh	140	5(3.57%)	0.5-6.64%	4(2.86%)	0.2-5.62%	3(2.14%)	0.26-4.54%	

#Level of significance determined based on the results of RBT; NS = not significant; * = significant at 5% level of significance (P value=0.013); 95% CI = 95% confidence interval.

ELISA, respectively. The prevalence of *Brucella* was higher in female than male (Table 3) which was not statistically significant (P value=0.683).

In the present study, higher prevalence of 5.0%, 4.17% and 3.33% by RBT, SAT and I-ELISA, respectively was reported in cattle of more than 4 years old in comparison to 3.75%, 2.5% and 1.25% in age group of ≥ 2-4 years and 3.33%, 1.67% and 1.67% in the age group of < 2 years by RBT, SAT and I-ELISA, respectively (Table 3). The difference among different age groups of cattle was statistically insignificant (P value=0.884).

A higher prevalence of *Brucella* was found in cattle with history of abortion that was 15%, 10% and 10% than without history of abortion that was 3.33%, 2.25% and 1.67% by RBT, SAT and I-ELISA, respectively (Table 3). There exists a statistically significant difference between prevalence of *Brucella* in cattle with history of abortion than without history of abortion (P value= 0.013).

In this study, the prevalence of *Brucella* in indigenous cattle was 4.67%, 3.33% and 2.67% in RBT, SAT and I-ELISA, respectively, while cross-bred cattle had a prevalence of 3.64%, 2.73% and 1.81% in RBT, SAT and I-ELISA, respectively (Table 3). This difference was not statistically significant (P value=0.683).

In this study, the highest prevalence of *Brucella* in cattle was found in Pabna district especially in female 5%, 3.33% and 2.5% compared to the prevalence of 3.57%, 2.86%, 2.14% in Mymensingh district as detected by RBT, SAT and ELISA, respectively. The difference in prevalence of *Brucella* in cattle between the two areas was not statistically significant (P value=0.568).

The overall seroprevalence of *Brucella* in cattle was 2.3% which is similar with the reports of Amin *et al*. (2004) and Rahman *et al*. (2006) who reported that the prevalence was 2.33%, 2% and 2.4%, respectively. The prevalence is lower than the finding of Nahar and Ahmed (2009) who reported 4.5% prevalence. This variation in the prevalence may be due to variation in the age, breed, sex, pregnancy status of the animal, study area, hygienic condition, breeding techniques, herd size, reproductive diseases and diagnostic tests applied (Kebede *et al*., 2008).

The comparison of the serological test result revealed a higher prevalence in RBT when compared to SAT and I-ELISA. The RBT showed more positive reaction to *Brucella* as compared to SAT and I-ELISA. Higher sensitivity and specificity of RBT was also reported by Muktaderul *et al*. (2011) and Muma *et al*. (2007), while Chakraborty *et al*. (2000) reported lower sensitivity but higher specificity of RBT.

The prevalence of *Brucella* was higher in female than male. This finding was similar to the findings recorded by Sharma *et al.* (2003). This may be due to presence of allantoic factors including erythritol, possibly steroid hormones and other substances in the female reproductive tract, especially in the gravid uterus which stimulate the growth of most of the *Brucellae* (Radolf, 1994). In the present study, higher prevalence was reported in cattle of more than 4 years old than in age group of ≥ 2-4 years and in the age group of < 2 years. This finding is coincided with the finding of Kazi *et al*. (2005). In contrast to the findings of the present study, Rahman *et al*. (2011a) reported the prevalence of brucellosis in the cows aged 2.5-4 years as 2·59%, while, in the cows over four years of age as 4·35%. Similarly, Amin *et al*. (2004) reported 2.3% and 4% prevalence in the < 4 and > 4 years age group, respectively. Age wise prevalence has also been studied by Abubakar *et al*. (2010) who showed that the incidence of brucellosis increased with age, and the incidence is high in sexually mature animals. The older animals are more susceptible to brucellosis due to more contact with infectious agents. Aged females suffering from malnutrition during pregnancy are more likely to be infected. Sergeant (1994) also found that there was no apparent association between age and serological status, or age and the prevalence. Ghani *et al*. (1998) and Uddin *et al*. (2007a, 2007b) stated that several factors such as age, sex, breed, location, herd size and living condition influence the seroprevalence of *Brucella.* It appears that the higher prevalence of brucellosis among older cows might be related to maturity with the advancing age. Thereby, the organism may have propagated to remain either as latent infection or it may cause clinical manifestation of the disease (Amin *et al*., 2005).

A higher prevalence of *Brucella* was found in cattle with history of abortion than without history of abortion. The present finding is in agreement with Rahman *et al*. (2006) who reported brucellosis to be higher in cattle with a history of abortion (15%) as compared to those with a history of returns to service (1·45%). In this study, the prevalence of *Brucella* in indigenous cattle was slightly more than cross-bred cattle. The difference

between the two groups was not statistically significant. Akbarmeher and Ghiyamirad (2011) reported that there exists differences in the prevalence of brucellosis in different breeds, but not statistically significant. In this study, the highest prevalence of brucellosis in cattle was found in Pabna district especially in female compared to the prevalence of *Brucella* in Mymensingh district.

Definitive diagnosis of brucellosis can be accomplished only through the direct demonstration and identification of the causative agent(s) by culture and isolation procedures (Orduña *et al*., 2000). But culture requires level 3 biological safety cabinet as the chance of laboratory personnel to be infected is high. Due to the lacking of laboratory facilities causative agent could not be isolated by culture. Further study is required for definitive diagnosis of brucellosis by highly sophisticated techniques like culture, PCR etc.

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Effects of Temperature Variation and Pellet Dimension on Settling Velocity of Fish Feed Pellets

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Abstract. In the present research, investigation was carried out for variation in settling velocity of some pelletized fish pellets in relation to floating time (T_f), diameter of pellets and temperature along with their water absorption properties under defined laboratory conditions. Among two diets of different ingredients DI and DII, it was observed that time for float (T_f) were greater at high range of temperature than lower range of temperature, for all tested pellets dimension (3 mm, 6 mm, 9 mm) of both diets DI and DII, while in case of settling velocity against high temperature range, lower values of settling velocities were recorded which shows an inverse relationship between them. On the other hand percent weight increments for diet DI were noted maximum for pellets size of 3,6 and 9 mm after 10 min of immersion i.e., 33.33, 55.55 and 38.46%, respectively, when compared to dry pellets.

Keywords: artificial fish feed, settling velocity, floating time, water absorption, water dispersion

Introduction

In order to maximize fish production and reduced waste dispersion, selection of ingredients, their composition and palletizing are of considerable importance. Modeling of waste dispersion is a key factor in regulation of rearing ponds. During feeding, significant amount of waste products (uneaten feed, fecal and soluble excretory material) are produced. Among these the primary reason for impairment of pond ecology is the settled uneaten feed pellets.These pellets not only affect over the benthos communities as well as other living biota (Vezzulli *et al.,* 2003; Beveridge *et al.,* 1991). Earlier studies suggested that 25-30% of dry weight of feed consumed is wasted as fecal matter (NCC, 1990; Butz and Vens-cappel, 1982). Decay of food matter could result in an accumulation of organic matter at pond bottom to manipulate the normal ecological conditions (Carroll *et al.,* 2003; Karakassis *at al.,* 2000). Keeping this in view, a number of models have been reviewed for monitoring the effects of temperature variation and pellet dimension on settling velocity and rate of soaking (Doglioli *et al.,* 2004; Cromey *et al.,* 2002; Perez *et al.,* 2002; Dudley *et al.,* 2000). It is true that settling velocity of uneaten feed pellets and soaking time is very useful tool to predict any model in intensive aqua culturing system. Earlier studies related with dimension of fish pellets involved in either sea water (Vassalo *et al.,* 2006; Chen *et al.,* 1999a) or fresh water (Elberizon and Kelly, 1998) have been recorded but in the present research instead of salinity, two temperature regimes are focused.

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Materials and Methods

In the present experiment two diets of different low cast ingredients more given to fish therefore, feed pellets of different length were produced. The proximate compositions of two feeds (D I & D II) are given in Table 1.

Measurement of settling velocity. Three different diameters (3 mm, 6 mm, 9 mm) of two different diets were examined at two ranges of temperature (28-30 °C and 20-22 °C) as described by Vassalo *et al.* (2006). Length was taken by the help of a vernier caliper. Plexiglas tube of 120 cm length with a diameter of 10 cm was used to find out the settling velocities of pellets following the method of Chen *et al.* (1999a). The tube was marked from the top, up to 5 cm for defining floating surface and the time to cover this distance was denoted as floating time (T_f). Then from this point, after every 50 cm, the tube was filled with fresh water and fixed vertically at different temperatures. 10 pellets of each length for each diet were examined. Pellets were gently dropped in water with the help of 0.01s chronometer. Time of pellet fall up to 5 cm (T_f) and beyond 5 cm to each 50 cm apart was noted. Water in the apparatus was changed for each

Table 1. Proximate composition of two feeds

Diet I		Diet II	
Ingredients	Percent values	Ingredients	Percent values
Rice polish	20	Rice protein	35
Rice bran	15	Corn gluten	30
Fish meal	20	Wheat bran	20
Sun flower meal	20	Fish meal	15
Wheat bran	10		
Bone meal	10		
Wheat flour	5		
Proximate values			
Crude protein	29	Crude protein	16.9
Fats	11.3	Fats	9.7
Moisture	5.7	Moisture	6.3

type of pellet and for temperature ranges. Temperature of water was noted by a thermometer and maintained at the required ranges by adding ice cubes.

Determination of water absorption property of pellets. The weight of feed pellet was not affected by change in temperature and salinity (Chen *et al.,* 1999b), so the water absorption property was recorded at room temperature, during the whole experiment. Ten pellets of each type of dimension were taken. After measuring their length and diameter, weight was taken in dried condition. All the selected pellets were soaked in fresh water for 2, 5 and 10 min of immersion time as indicated by Vassalo *et al.* (2006). After passing the immersion period pellets were taken out from water and left on absorbing paper for absorption of excess water. Finally all pellets were measured and weighed again to observe the changes in pellet dimension and weight.

Results and Discussion

Settling velocity. The effects of temperature variation and pellets dimension on T_f and V_{set} are presented in Tables 2-3. Keeping temperature as a controlling factor, it was observed that time for float (T_f) of both diets DI and DII were greater at high range of temperature than lower range of temperature for all tested pellets dimensions (3 mm, 6 mm, 9 mm). On the other hand the settling velocity (V_{set}) did not respond as T_f i.e., against high temperature lower V_{set} recorded when compared to lower range of temperature. Furthermore, it is attributed that there was an inverse relationship between T_f and V_{set} for all dimensions of pellets (Fig. 1- 4).

Statistically it is proven by general linear model (GLM). Analysis of variance for floating time (Tables 4-5) indicated significant differences ($P<0.05$) for pellets dimension within each temperature regime (28-30 °C, 20-22 °C). The interaction between pellets dimension and temperature regimes was significantly affected over time for floating pellets on water surface. Tables 6-7 show response of pellets in terms of settling velocity (V_{set}) (Fig. 5-8). Again a highly significant difference was noted for pellets dimension and temperature regimes ($P< 0.05$), however,

Table 2. Settling velocity (V_{Set}) and floating time (T_f) for three different dimensions of fish feed pellets of DI (3.5 mm, diameter) with reference to two temperature regimes

S.No.	Temperature (28-30 ºC)						Temperature (20-22 ºC)					
	3 mm		6 mm		9 mm		3 mm		6 mm		9 mm	
	T_f (sec)	V_{set} (m/s)	T_f (sec)	V_{set} (m/s)	T_f (sec)	V_{set} (m/s)	T_f (sec)	V_{set} (m/s)	T_f (sec)	V_{set} (m/s)	T_f (sec)	V_{set} (m/s)
1	1.2	0.071	1.1	0.095	0.52	0.098	0.63	0.079	0.75	0.082	0.49	0.113
2	1.68	0.068	1.02	0.085	0.47	0.089	0.61	0.08	0.42	0.107	0.51	0.107
3	1.56	0.077	0.8	0.087	0.4	0.1	0.71	0.078	0.7	0.092	0.35	0.097
4	1.36	0.08	0.6	0.081	0.42	0.094	0.4	0.087	0.73	0.083	0.37	0.092
5	1.52	0.079	0.56	0.094	0.7	0.094	0.45	0.079	0.51	0.097	0.32	0.106
6	1.62	0.081	0.89	0.092	0.46	0.104	0.7	0.087	0.54	0.105	0.27	0.092
7	1.23	0.069	0.92	0.088	0.64	0.095	0.51	0.093	0.43	0.1	0.39	0.094
8	1.38	0.09	1.02	0.087	0.38	0.104	0.77	0.075	0.6	0.102	0.41	0.118
9	1.6	0.071	1.96	0.085	0.59	0.105	0.74	0.082	0.37	0.104	0.53	0.123
10	1.51	0.084	0.72	0.08	0.62	0.096	0.59	0.076	0.36	0.133	0.46	0.098
Mean	1.46 (0.16)	0.077 (0.007)	0.95 (0.39)	0.16 (0.24)	0.52 (0.11)	0.097 (0.005)	0.61 (0.12)	0.087 (0.005)	0.54 (0.14)	0.104 (0.01)	0.41 (0.08)	0.104 (0.01)

Values in subscripts are standard deviations.

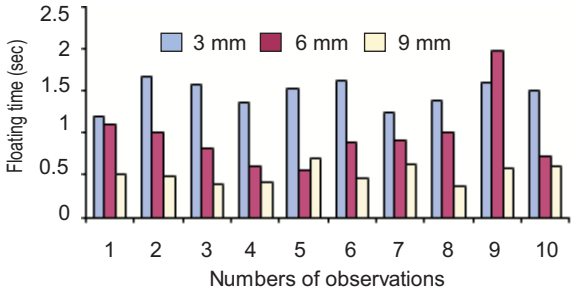

Fig. 1. Floating time of three different dimensions of fish feed pellets of DI at 28-30 ºC.

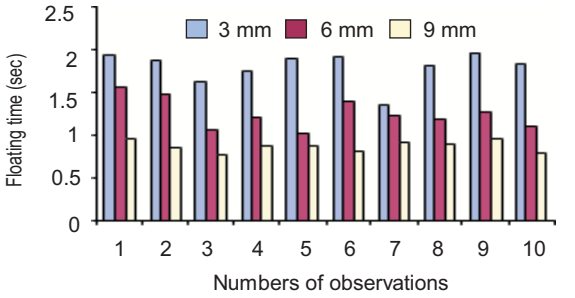

Fig. 5. Floating time of three different dimensions of fish feed pellets of DII at 28-30 ºC.

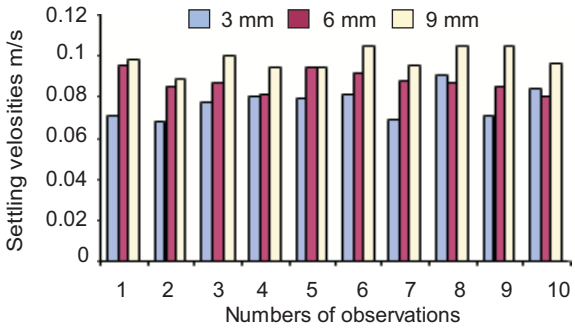

Fig 2. Settling velocitie of three different dimensions of fish feed pellets of DI at 28-30 °C.

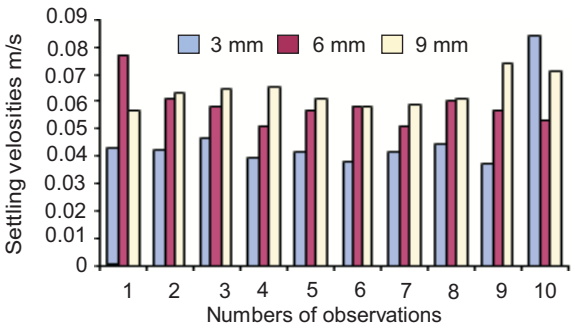

Fig. 6. Settling velocities of three different dimensions of fish feed pellets of DII at 28-30 °C.

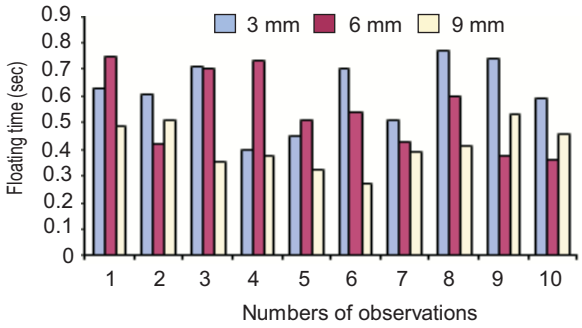

Fig 3. Floating time of three different dimensions of fish feed pellets of DII at 20-22 ºC.

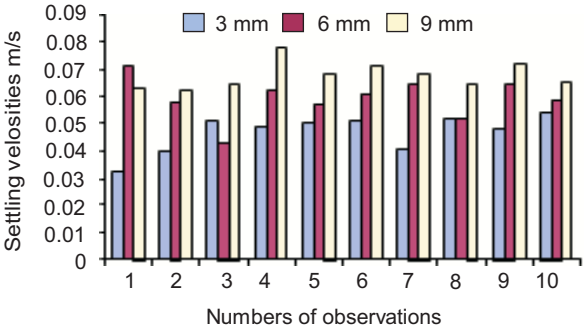

Fig. 7. Settling velocities of three different dimensions of fish feed pellets of DII at 20-22 ºC.

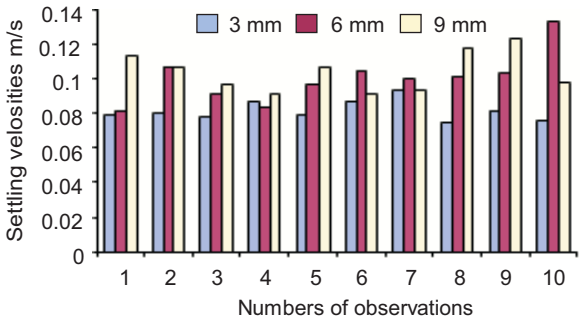

Fig 4. Settling velocities of three different dimensions of fish feed pellets of DII at 20-22 °C.

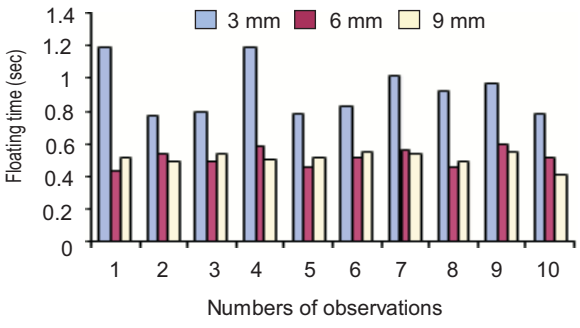

Fig. 8. Floating time of three different dimensions of fish feed pellets of DII at 20-22 °C.

Table 3. Settling velocity (V_{set}) and floating time (T_f) for three different dimensions of fish feed pellets DII (2mm, diameter) with reference to two temperature regimes

S.No	Temperature 28-30 ºC						Temperature 20-22 ºC					
	3 mm		6 mm		9 mm		3 mm		6 mm		9 mm	
	T_f (sec)	V_{set} (m/s)	T_f (sec)	V_{set} (m/s)	T_f (sec)	V_{set} (m/s)	T_f (sec)	V_{set} (m/s)	T_f (sec)	V_{set} (m/s)	T_f (sec)	V_{set} (m/s)
1	1.93	0.043	1.56	0.077	0.96	0.057	1.19	0.032	0.43	0.071	0.51	0.063
2	1.86	0.042	1.48	0.061	0.84	0.063	0.77	0.04	0.54	0.058	0.49	0.062
3	1.63	0.047	1.06	0.058	0.77	0.064	0.8	0.051	0.49	0.043	0.54	0.064
4	1.74	0.039	1.2	0.051	0.86	0.065	1.19	0.049	0.59	0.062	0.5	0.078
5	1.89	0.041	1.02	0.057	0.87	0.061	0.78	0.05	0.46	0.057	0.51	0.068
6	1.91	0.038	1.4	0.058	0.81	0.058	0.83	0.051	0.52	0.061	0.55	0.071
7	1.34	0.041	1.23	0.051	0.92	0.059	1.01	0.041	0.56	0.064	0.54	0.068
8	1.82	0.045	1.19	0.06	0.89	0.061	0.92	0.052	0.46	0.052	0.49	0.064
9	1.96	0.037	1.27	0.057	0.96	0.074	0.96	0.048	0.6	0.064	0.55	0.072
10	1.83	0.084	1.1	0.053	0.79	0.071	0.78	0.054	0.51	0.059	0.41	0.065
Mean	1.79 (0.18)	0.041 (0.01)	1.25 (0.17)	0.058 (0.007)	0.86 (0.06)	0.063 (0.005)	0.92 (0.16)	0.046 (0.006)	0.51 (0.05)	0.059 (0.007)	0.50 (0.04)	0.067 (0.004)

Values in subscripts are standard deviations.

Table 4. Two way analysis of variance for floating time of diet DI

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Pellets	2	3.2891	3.2891	1.6445	40.93	0.000
Temperature	1	3.1878	3.1878	3.1878	79.33	0.000
Pellets × Temperature	2	1.4014	1.4014	0.7007	17.44	0.000
Error	54	2.1699	2.1699	0.0402	-	54
Total	59	10.0482	-	59	-	-

DF = degree of freedom; Seq SS = sequential sum of square; Adj SS = adjusted sum of square; MS = means of square; F = F ratio; P = probability ratio.

Table 5. Two way analysis of variance for settling velocity of diet DI

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Pellets	2	0.0048632	0.0048632	0.0024316	31.09	0.000
Temperature	1	0.0009362	0.0009362	0.0009362	11.97	0.001
Pellets × Temperature	2	0.0002077	0.0002077	0.0001039	1.33	0.274
Error	54	0.0042231	0.0042231	0.0000782	-	-
Total	59	0.0102302	-	-	-	-

DF = degree of freedom; Seq SS = sequential sum of square; Adj SS = adjusted sum of square; MS = means of square; F = F ratio; P = probability ratio.

the interaction between pellet size and temperature regimes did not significantly affect over V_{set}.

Water absorption property of pellets. Table 8 shows immersed pellets weight increment with reference to time of immersion i.e., 2, 5 and 10 min. None of the pellets of diet DI exhibit any change in dimension after three different times of immersion. However, in case of diet DII 3 mm size pellets were dissolved or loosed their dimension when immersed for 5 and 10 min due to having small diameter than diet DI. On the other hand percent weight increments for diet DI were noted maximum for pellets size of 3,6 and 9 mm after 10 min of immersion i.e., 33.33, 55.55 and 38.46%, respectively, when compared to dry pellets and 2 and 5 min of immersion time. Totally different trends were observed for diet DII in this context. With comparison

to dry pellets weight increment of 100%, 50% and 66.66% were recorded for 3, 6 and 9 mm of pellets size, respectively, after 2 min of immersion.

The same increasing pattern of weight enhancement was noted for DII pellets having the same pellet size i.e., 100% after 5 min and 150% after 10 min. The differences between the weight increment values of DI and DII showed that as the diameter of pellets increases, their water absorption property decreases (Chen *et al.,* 1999a). It was also noted that more or

Table 6. Two way analysis of variance for floating time of diet DII

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Pellets	2	4.0049	4.0049	2.0024	66.43	0.000
Temperature	1	5.7722	5.7722	5.7722	191.49	0.000
Pellets × Temperature	2	0.5189	0.5189	0.2594	8.61	0.001
Error	54	1.6277	1.6277	0.0301	-	-
Total	59	11.9237	-	-	-	-

DF = degree of freedom; Seq SS = sequential sum of square; Adj SS = adjusted sum of square; MS = means of square; F = F ratio; P = Probability ratio.

Table 7. Two way analysis of variance for settling velocity of diet DII

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Pellets	2	0.00476	0.0047	0.0023805	61.01	0.000
Temperature	1	0.0001908	0.0001908	0.0001908	4.89	0.031
Pellets × Temperature	2	0.0000492	0.0000492	0.0000246	0.63	0.536
Error	54	0.0021071	0.0021071	0.0000390	-	-
Total	59	0.0071082	-	-	-	-

DF = degree of freedom; Seq SS = sequential sum of square; Adj SS = adjusted sum of square; MS = means of square; F = F ratio; P = probability ratio.

Table 8. Mean weight increase (%) of pellets of DI and DII as a function of different immersion times (2, 5 and 10 minutes)

Before immersion																	
Diet I									Diet II								
L(mm)			W(gm)						L(mm)			W (gm)					
3			0.6						3			0.1					
6			0.9						6			0.2					
9			1.3						9			0.3					
After immersion																	
Time in minutes																	
2			5			10			2			5			10		
L	W	MWI	L	W	MWI	L	W	MWI	L	W	MWI	L	W	MWI	L	W	MWI
3	0.7	16.66	3	0.8	25	3	0.8	33.33	3	0.2	100	3	0.2	100	3	0.1	**
6	1.2	33.33	6	1.3	44.44	6	1.4	55.55	6	0.3	50	6	0.4	100	6	0.5	150
9	1.4	7.69	9	1.7	30.76	9	1.8	38.46	9	0.5	66.66	9	0.6	100	9	0.6	100

L, length; W = weight; MWI = mean weight increase (%), ** = dissolved completely.

less all under observed pellets of diet DII were dissolved or disintegrated into its constituents revealing greater absorption properties as compared to diet DI. The role of formulated diets definitely contributes in rate of production. Feed manufacturers have diverted their efforts towards the physical qualities including settling velocity and soaking or immersion time. According to linear law of stokes, a particle falls in water with its settling velocity with respect to its dimension, density and viscosity. Among these, the viscosity is highly influenced by temperature, solute concentration and hydrostatic pressure. In present feed trial smaller pellets size of diet DII (3 mm) were dissolved or loosed their dimension when immersed for 5 and 10 min while more pellets of diet DI show any change in dimension after three different times of immersion. These results were in line with the findings of Thomas and Vander Poel (1996), who claimed that small diameter pellets (3 mm) were found to be more susceptible to breakage than larger diameter pellets (6 mm). The differences between diet DI and DII can be attributed to variations in formulation because of the water soaking ability of different ingredients. It shows that the diet DII is more friable than diet DI. Doglioli *et al.* (2004) focused on behaviour of pallets made for salmon aquaculture and potentially applied and described a model.

In present research, a comparison was undertaken between two diets DI and DII to investigate the settling velocity and time for immersion. The findings were indicated that an inverse relationship exist between T_f and V_{set} for all dimensions of pellets. As far as immersion time is concerned (2, 5 and 10 min) none of the pellets of diet DI exhibit any change in dimension after three different times of immersion. However, for diet DII 3 mm sized pellets were dissolved when immersed for 5-10 mins due to smaller in size. These results conclude that two diets have no similar pattern of T_f and V_{set}, although Wood (1987) found a relationship between pellet hardness and friability. Relationship between the under observed parameters are generally only found where the feed ingredients and pellet producer are same as suggested by Thomas and Vander Poel (1996).

The outcome from Tables mean velocities to sink for diet DI (3 mm) were 0.077 m/s in water having two temperature ranges followed by 0.087 m/s. 0.16 m/s and 0.100 m/s for 6 mm and 0.097 m/s and 0.104 m/s for 9 mm respectively.

For pellets size of 3 mm of diet DII 0.041 m/s, 0.046 m/s were calculated with the increasing trend for 6 mm, 9 mm i.e., 0.058 m/s, 0.059 m/s and 0.063 m/s,0.067 m/s, respectively. When comparing these results with the results of earlier studies the similar attributions are found.

Gowen *et al.* (1989) quoted results from unpublished data of velocities of 0.09 to 0.15m/s and used a settling velocity equal to 0.12 m/s in developing waste dispersion models. Findlay and watling (1994) provided data on several North America pellet types or sizes and quoted settling rates of 0.055 m/s and 0.155 m/s for 3 mm and 10 mm dry pellets, respectively. Elberizon and Kelly (1998) showed settling velocities of freshwater salmonid pellet diets ranging from 0.05 to 0.12 m/s for 2 mm and 8 mm pellet sizes, respectively.

The floating time since the ANOVA test showed that it significantly affects settling velocity. The reason for this fact may be because of the observed weight increment of pellets immersed in the water at the surface before they start to fall. The soaking experiment provides a quantitative estimate of this process, pointing out that the phenomenon is greater for smaller particles. Thus, it could be said that the influence of temperature and salinity on the settling velocity is indirect via T_f the lesser the percentage of uneaten feed. However, a quantitative calculation of this link is very hard to achieve but knowing the T_f value provides a valuable piece of information for model calibration and validation processes.

Finally, the present study provides important information for aquaculture wastes dispersion modeling. A realistic dispersion model would then have to consider: (a) the diameter of the actual feed distributed to fishes: (b) the seasonal variation of temperature. Collaboration with farmers, nutritional data collection and hydrological measurements will be useful to improve aquaculture impact predictions. Two temperature ranges show the seasonal temperature variations which have a significant influence or the settling velocity and floating time.

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Short Communication

Antagonistic Activity of Bacterial Strains Isolated from Human Producing Biological Control Agents

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Abstract. In present research the antagonistic activity of human bacterial pathogens *viz*., *Streptococcus pyogenes, Staphylococcus epidermidis, Klebsiella pneumonia, Staphylococcus aureus, Serratia marcescens, Escherichia coli* and *Pseudomonas aeruginosa* were analysed. Significant amount of lactic acid production was shown by *S. pyogenes, S. epidermidis* and *P. aeruginosa* (63 mg/mL, 54 mg/mL, and 54 mg/mL), while *S. pyogenes* and *S. epidermidis* also produced significant quantity of hydrogen peroxide (1.70 mg/mL and 1.605 mg/mL). An impressive diversity of spots of chemical constituents was also obtained through thin layer chromatography (TLC) from broth culture of bacterial strains. The antagonistic activity may be indicated the potency of lactic acid and hydrogen peroxide production to inhibit the microbes.

Keywords: lactic acid, hydrogen peroxide, antagonistic activity, biological control agent

Microorganisms are not only the cause of infections; they can also produce organic substances that can cure infections (Jensen and Fencial, 2000) and various bioagents have been isolated from bacteria, fungus, and algae (Abdel-Fattah *et al*., 2011; Galal *et al*., 2011). Screening of bioactive compounds is based on some major factors like selection of a proper microorganism, isolation and culture methods and the detection and identification of their metabolites (Alwathnani and Perveen, 2012; Reddy *et al*., 2011).

Seven bacterial strains i.e., *Steptococcus pyogenes*, *Staphylococcus epidermidis*, *Klebsiella pneumonia*, *Staphylococcus aurues*, *Serratia marcescens*, *E. coli* and *P. aeruginosa* were isolated from different clinical samples of human. Most of the strains were catalase positive and indole negative (Awan *et al*., 2013). Antagonistic activity of human isolated pathogens was analysed through three agar disc diffusion systems such as phosphate buffer culture disc (PBCD) method, culture agar disc (CAD) method, and cell free supernatant disc (CFSD) method (Drummond and Waigh, 2000; Colle and Marr, 1989), zone of inhibition was measured in mm (Fig. 1).

Trivedi *et al*. (2008) and Trivedi and Sa (2008) demonstrated the antagonistic activity of *Pseudomonas* spp. against two phytopathogenic fungi, *Fusarium oxysporium* and *Alternaria alternata*. Similarly, in current research *P. aureginosa* results indicated that PBCD is much better method than rest of two methods. It has been analysed that *P. aureginosa* showed maximum activity against *S. pyogenes*, moderately high activity against *S. epidermidis* and moderately low against all other tested pathogens, respectively (Fig. 1A). Present results are consistent with Sindhu and Dadarwal (2001). The obtained results of TLC were found similar to that detected by Kumar *et al*. (2005) (Table 1).

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Table 1. Thin layer chromatography of microbes by using various solvent systems

Human microbes	UAW 1	UAW 2	UAW 3	UAW 4	UAW 5
Pseudomonas aeruginosa	S1= 0.211 S2= 0.923	S1= 0.961 S2= 0.980	no result	S1= 0.608	no result
Staphyloccocus epidermidis	S1= 0.063 S2= 0.80 S3= 0.87	no result	no result	S1= 0.225	no result
Staphylococcus aureus	S1= 0.319 S2= 0.382 S3= 0.531	no result	S1= 0.833	S1= 0.394	S1= 0.969
Klebsiella pneumonia	no result S2= 0.292 S3= 0.365	no result	S1= 0.121	no result	S1= 0.6
Escherichia coli	no result	S1= 0.971	S1= 0.0444	S1= 0.4	no result
Streptococcus pyogenes	no result S2= 0.680	S1= 0.319	no result	no result	no result
Serratia marcescens	S1= 0.088 S2= 0.888	S1= 0.782	no result	no result	S1= 0.875

Note: spots on TLC indicated by S1, S2, S3 and solvent systems were labeled as UAW 1; EtoAc: EthoH 9:1; UAW 2: EtoAc: MeoH 4:1; UAW3: EtoAc: Pet.ether 4:1; UAW 4: EtoAc: Pet.ether 1:4; UAW5: Hex: Acetone 7:3.

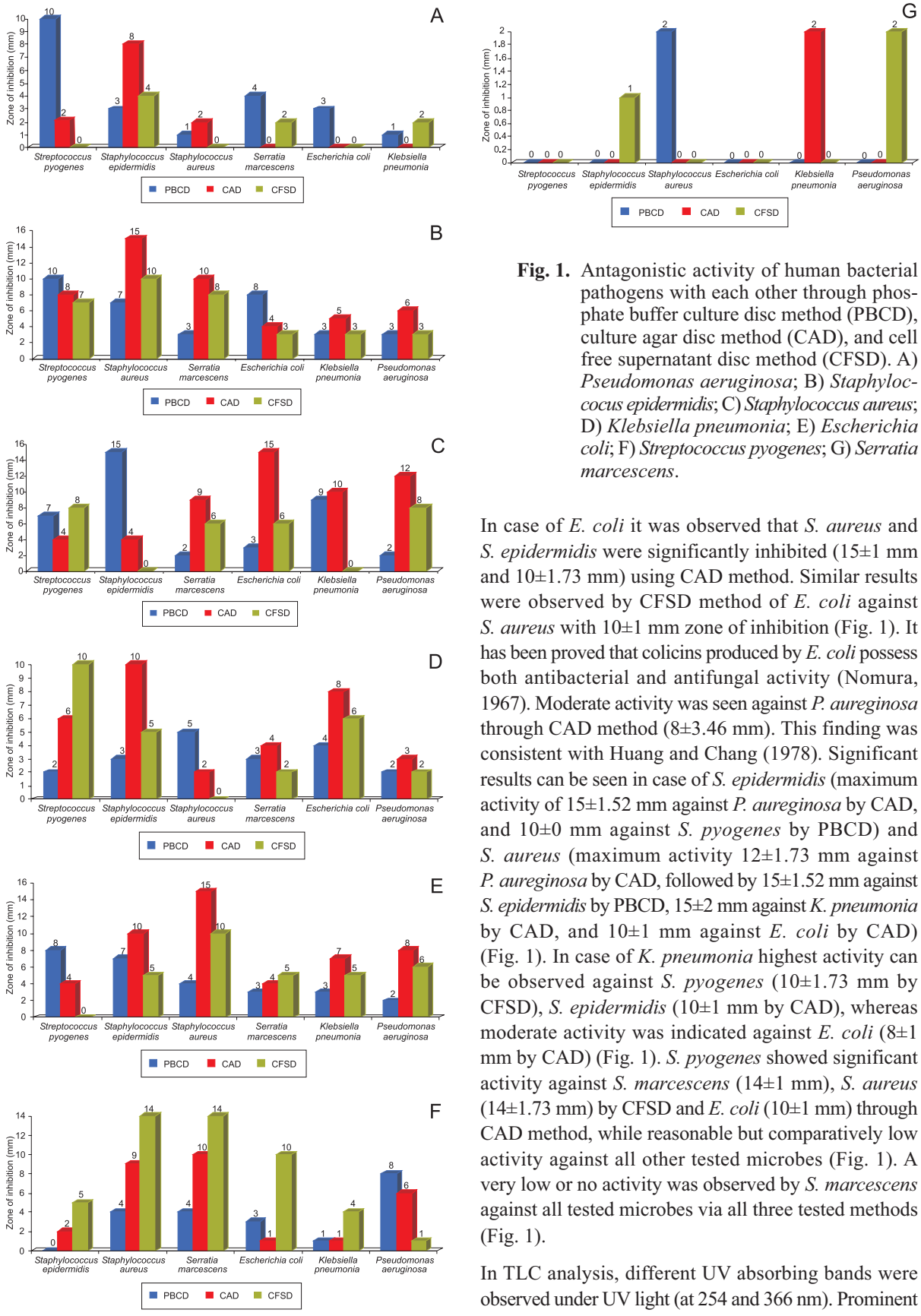

Fig. 1. Antagonistic activity of human bacterial pathogens with each other through phosphate buffer culture disc method (PBCD), culture agar disc method (CAD), and cell free supernatant disc method (CFSD). A) *Pseudomonas aeruginosa*; B) *Staphylococcus epidermidis*; C) *Staphylococcus aureus*; D) *Klebsiella pneumonia*; E) *Escherichia coli*; F) *Streptococcus pyogenes*; G) *Serratia marcescens*.

In case of *E. coli* it was observed that *S. aureus* and *S. epidermidis* were significantly inhibited (15±1 mm and 10±1.73 mm) using CAD method. Similar results were observed by CFSD method of *E. coli* against *S. aureus* with 10±1 mm zone of inhibition (Fig. 1). It has been proved that colicins produced by *E. coli* possess both antibacterial and antifungal activity (Nomura, 1967). Moderate activity was seen against *P. aureginosa* through CAD method (8±3.46 mm). This finding was consistent with Huang and Chang (1978). Significant results can be seen in case of *S. epidermidis* (maximum activity of 15±1.52 mm against *P. aureginosa* by CAD, and 10±0 mm against *S. pyogenes* by PBCD) and *S. aureus* (maximum activity 12±1.73 mm against *P. aureginosa* by CAD, followed by 15±1.52 mm against *S. epidermidis* by PBCD, 15±2 mm against *K. pneumonia* by CAD, and 10±1 mm against *E. coli* by CAD) (Fig. 1). In case of *K. pneumonia* highest activity can be observed against *S. pyogenes* (10±1.73 mm by CFSD), *S. epidermidis* (10±1 mm by CAD), whereas moderate activity was indicated against *E. coli* (8±1 mm by CAD) (Fig. 1). *S. pyogenes* showed significant activity against *S. marcescens* (14±1 mm), *S. aureus* (14±1.73 mm) by CFSD and *E. coli* (10±1 mm) through CAD method, while reasonable but comparatively low activity against all other tested microbes (Fig. 1). A very low or no activity was observed by *S. marcescens* against all tested microbes via all three tested methods (Fig. 1).

In TLC analysis, different UV absorbing bands were observed under UV light (at 254 and 366 nm). Prominent

coloured bands were observed by staining with anisaldehyde/H_2SO_4. The most promising diversity of coloured spots was seen in the crude extracts of three pathogens.

All the tested strains produced considerable amount of lactic acid and comparatively little amount of hydrogen peroxide (Table 2). This study revealed that the antagonistic activity perhaps indicated the potency of lactic acid and hydrogen peroxide production to inhibit the microbes.

Table 2. Estimation of biological control compounds production by microbes

Bacterial isolates	Production of control agents (mg/mL)	
	Lactic acid	Hydrogen peroxide
Pseudomonas aeruginosa	54	0.535
Staphyloccocus epidermidis	54	1.605
Staphylococcus aureus	9	0.535
Klebsiella pneumonia	7.2	1.070
Escherichia coli	1.8	1.070
Streptococcus pyogenes	63	1.70
Serratia marcescens	2.7	1.070

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Short Communication

Effect of Arthropods Abundance on the Red Junglefowl Population in Oil Palm Plantation Habitat

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Abstract. The study was conducted for one year in the 4-year and 8-year old oil palm plantation at Sungai Sedu Estate, Selangor, Malaysia, to observe whether the abundance of arthropods affects the density of red junglefowl (*Gallus gallus spadiceus*). The arthropods were collected by three methods i.e., litter, pitfall and sweep net. The results indicated that the arthropods abundance in both the study areas was found to be almost similar. It is suggested that arthropods abundance has little effect on the density of red junglefowl in oil palm plantation.

Keywords: arthropods, red junglefowl density, oil palm plantation, *Gallus gallus spadiceus*

The red junglefowl (order Galliformes) is referred as the ancestor bird of local poultry (Darwin, 1875). It is distributed throughout India, Burma, South China, Malaya, Sumatra, Philippines Islands, Fiji and New Guinea (Delacour, 1977). In Peninsular Malaysia, its sub species *Gallus gallus spadiceus* is found up to the elevation of 1676 m (Yatim, 1993), and most of its populations inhabit agriculture areas such as oil palm, rubber and tea plantation (Arshad and Zakaria, 2009; Azhar *et al*., 2008; Zakaria *et al*., 2003; Abdullah and Babjee, 1982; Davison and Scriven, 1987). It is highly opportunistic and omnivorous in diet (Collias and Collias, 1967) and takes a wide variety of insects particularly teremites and ants (Medway and Well, 1976).

This study was undertaken to determine whether arthropods abundance has any effect on the density of red junglefowls in different aged oil palm plantation.

The study of arthropod abundance was conducted from August 1996 to July 1997 at Sungai Sedu Oil Palm Estate, Banting, Selangor, Malaysia in the 4 year and 8 year old oil palm plantation. Three methods namely litter collection pitfall traps and sweep net were used for the sampling of arthropods (Southwood, 1978).

Arthropods in litter. Litter samples were collected systematically. Five plots, 30x30 m, were selected randomly and marked. In each plot, four samples were collected monthly. Sample was taken at random by placing a 0.25 m^2 wooden frame on the ground. The litter inside each square was collected up to 1 cm soil depth and samples were collected into plastic bags.

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Pitfall traps. Uncapped glass bottles of 8.5 cm mouth diameter and 7.5 cm deep were buried in the ground at random with their open tops flush to the litter surface. Bottles were filled to a depth of 5 cm with water and then covered with a piece of plywood raised from about 15 to 18 cm above the bottle to prevent the entry of rain water. Sorbic acid was used as preservative at the rate of one gram per sample. The bottles were examined after seven days. The sample insect collections were preserved in 70% ethanol.

Sweep net. Twenty strips, 30 m long and 1 m wide were selected randomly in both study areas. Ten sweeps were taken in each strip through the upper layer of vegetation and considered as one sample. Contents of sweep net were placed in ethylacetate kill jar until the arthropods were dead, then the insect material was transferred to labeled plastic bottle and preserved in 70% ethanol. Samples were not taken during drizzling or immediately after rain.

Data analysis. Arthropod abundance was defined as number of arthropods per sample. Data of arthropods abundance obtained by all trapping methods were pooled. Student's t-test analysis (Steel and Torrie, 1980) was used to detect the difference of abundance of arthropods between study sites. The eight orders of arthropods i.e., Hymenoptera (Formicidae), Orthoptera, Coleoptera, Hemiptera, Isoptera, Dermaptera, Arachnida and Isopoda that were considered to be important food sources for red junglefowl (Arshad *et al*., 2000). Student's t-test was also used to determine the difference of abundance of insects in 4 year and 8 year old oil palm plantations. Published data of earlier similar study

on population density of red junglefowl by Zakaria *et al*. (2003) was reviewed for comparison with arthropods abundance. The results were declared significant at P=0.05. All statistical analyses were performed by using Statistical Analysis System software (SAS, 1990).

The total number of arthropods caught in 4 year old oil palm plantation was 15872, whereas total of arthropods counted in 8 year old oil palm plantation were 14616 (Table 1). The results indicated that there was no significant variation in the abundance of arthropods caught in both study areas (t=1.41, P>0.05; Fig. 1). The eight orders that were considered to be main food items for red junglefowl were also found in the same abundance in both study areas (t=0.12, P>0.05; Fig. 2).

Zakaria *et al*. (2003) reported that the density of ref junglefowl in the 4 year old oil palm plantation was 84.22±5.45/km^2 while in the 8 year old oil palm plantation was 27.80±3.57/km^2. This indicated that the population density of red junglefowl did not depend on arthropods. This is because even though the abundance of arthropods in the two areas was about the same, the density of red junglefowl was higher in the 4 year old oil palm plantation. There may be other factors that affect the density of red junglefowl. Zakaria *et al*., 2003 reported that canopy cover significantly affects the density of red junglefowl.

The red junglefowls are opportunist feeders in the oil palm habitat, i.e. plant materials (80.88%) as well as

Table 1. Abundance of arthropods by different methods in 4 year and 8 year old oil palm plantation at Sungai Sedu Estate

Arthropods	4 Year old oil palm plantation			8 Year old oil palm plantation		
	Pitfall trap	Litter analysis	Sweep net	Pitfall trap	Litter analysis	Sweep net
Insecta						
Coleoptera	552	551	247	604	695	259
Collembola	530	276	16	1041	280	1
Dermaptera	114	157	1	75	-	1
Diplura	-	-	-	-	5	-
Diptera	181	7	194	132	-	156
Hempitera	15	10	194	17	4	72
Homoptera	5	10	367	6	-	410
Hymenoptera (Formicidae)	4581	1791	1062	4673	979	1168
Hymenoptera(Others)	13	33	51	14	-	67
Isoptera	38	3	3	62	-	1
Lepidoptera	44	11	64	35	5	58
Neuroptera	8	1	-	6	2	-
Odonata	-	-	9	-	-	-
Orthoptera	1021	325	561	709	179	594
Psocoptera	-	3	-	-	7	1
Thysanoptera	1	-	-	2	-	-
Unidentified insects	3	48	4	38	21	1
Chilopoda	81	32	1	39	9	-
Diplopoda	19	48	-	2	1	-
Crustacea						
Amphipoda	234	9	-	114	1	-
Isopoda	22	22	-	7	4	-
Arachnida						
Acarina	116	321	5	21	61	-
Araneida	489	95	1254	570	73	1231
Chelonethida	-	2	-	-	-	-
Phalangida	7	9	1	-	2	1
Total	8074	3764	4034	8167	2328	4121
G. Total		15872			14616	

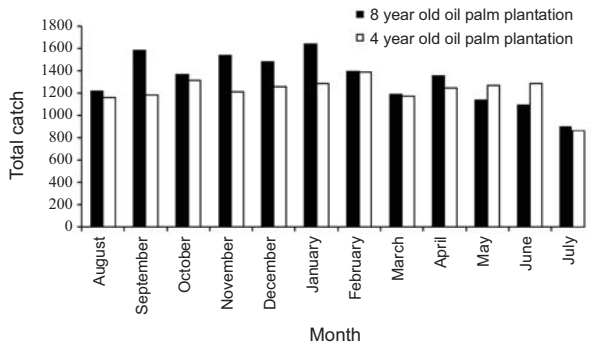

Fig. 1. Monthly catch trend of arthropods in the 4 year and 8 year old oil palm plantations at Sungai Sedu Estate.

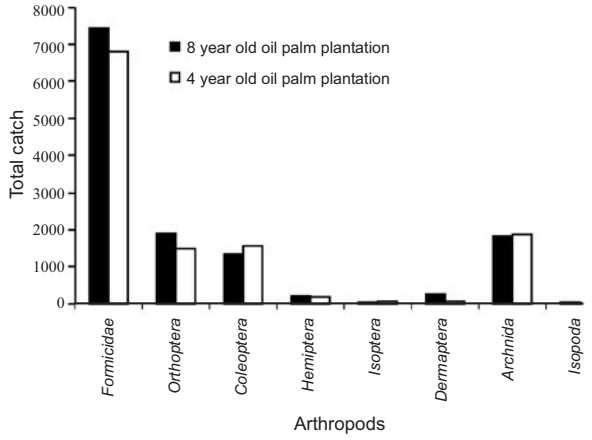

Fig. 2. Main arthropods considered as food for red junglefowl in the 4 year and 8 year old oil palm plantations at Sungai Sedu Estate.

animal materials (19.12%) (Arshad *et al*., 2000). Although the findings of this study showed that the population of red junglefowl did not depend upon the arthropod abundance but many studies highlighted the importance of arthropods in the diet of galliformes. Arthropods are also important food for ruffed grouse (*Bonasa umbellus*) chicks. The diet of chicks less than three weeks of age is more than 90% invertbrates, and these are dominant in their diet for about five weeks after hatching (Kimal and Samuel, 1984). Therefore, arthropods might be important food sources for red junglefowl but might not be sufficient to regulate the red junglefowl population size. Other factors such as suitability of habitats and plant food sources might also affect its abundance.

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Review

Nitrogen Fertilizer Management Strategies for Rice Production in Bangladesh

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Abstract. Various ways of increasing nitrogen (N) fertilizer use efficiency in rice culture has been evaluated by conducting field experiments at Bangladesh Rice Research Institute (BRRI). Nitrogen fertilizer use efficiency in rice culture can be increased by root-zone application of modified forms of urea like urea large granule (ULG) and urea super granule (USG). The efficiency of urea-N can be increased to some extent even by injection of the conventional prilled urea (PU) into the root-zone of the rice plant by the instrument "Pneumatic Urea Injector". Application of sulphur (S) along with N increases N use efficiency in rice culture in S deficient soils. Modern rice varieties having relatively taller plant stature can exploit more soil N for grain production compared to short statured varieties. Different varieties require different amounts of N for maximum grain yield and it is important to note it to avoid indiscriminate application of a single N rate for all the varieties.

Keywords: nitrogen fertilizer, rice production, environmental pollution control

Rice is the major cereal crop in Bangladesh. With increase in population, the demand for rice is increasing over the years. With intensive research rice yield per unit area has increased gradually over the years (Table 1). Consequently fertilizer consumption especially N fertilizer has also been increased gradually (Table 2). Nitrogen (N) is a primary nutrient for all crops. Rice crop requires large amount of N for its growth, development and grain production (Sahrawat, 2000). Generally rice plant removes around 14-20 kg N to produce one tonne of rough rice including straw (Table 3). Most of the rice soils of the world are deficient in N, and biological nitrogen fixation by Cyanobacteria and diazotrophic bacteria can only meet a fraction of the N requirement (Sattar *et al*., 2008; Hashem, 2001; Baldani *et al*., 2000). Thus, fertilizer N application is essential to meet the crop requirement. But, the efficiency of added fertilizer N in rice culture depends on N sources, application method, rate of N as well as management practices as evidenced by the ^{15}N tracer studies (Wang *et al*., 2011; Chen *et al*., 2010; Wang *et al*., 2008). Prilled urea (PU) is applied as N source for rice but the efficiency of added N from PU is very low, generally it is around 30-45% and in many cases even much lower as determined by the ^{15}N tracer technique (Table 4). This low N use efficiency in rice culture is attributed mainly to denitrification, ammonia volatilisation and leaching losses (Hakeem *et al.*, 2011; Mai *et al.*, 2010; Zhao *et al.*, 2010; Choudhury and Kennedy, 2005). Ammonia volatilisation and denitrification cause atmospheric pollution through the production of greenhouse gases like nitrous oxide, nitric oxide and ammonia (Choudhury and Kennedy, 2005; Reeves *et al*., 2002). Nitrous oxide absorbs infrared radiation and depletes the stratospheric ozone layer. Nitric oxide causes acid deposition by forming nitric acid. Leaching causes nitrate toxicity in the groundwater. High nitrate toxicity in the groundwater causes human health problems (Shrestha and Ladha, 1998). These problems are of great concern to the agronomists, soil and environmental scientists and policy makers around the world. Appropriate strategies should be taken to reduce N losses and thereby minimize these environmental problems. In this regard, research has been conducted around the world in several research organizations including Bangladesh Rice Research Institute (BRRI). This paper reviews some salient findings of BRRI already published in different journals to accumulate the information altogether indicating N fertilizer management strategies for sustainable rice production and control of environmental pollution problems.

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Table 1. Population, rice cropped area, rice production and yield in Bangladesh over the years since 1960

Year	Population (million)	Rice cropped area (000 ha)	Paddy production (000 tonnes)	Paddy yield		Rice production (kg/person)	Rice available excluding import (g/person/day)
				t/ha	kg/person		
1960	51.585	8857	14522	1.64	282	197	540
1970	66.671	9912	16731	1.69	251	176	481
1980	88.219	10309	20844	2.02	236	165	453
1990	109.820	10435	26781	2.57	244	171	468
2000	129.194	10887	37633	3.46	291	204	559
2010	153.437	11800	48455	4.11	316	221	606

The population data was collected from the website http://www.populstat.info/Asia/bangladesh.htm. The data on rice cropped area and paddy (un-milled rice) production have been collected from the USDA database available in the IRRI website (IRRI, 2011). Paddy yield per person was converted to rice (milled rice) yield considering 70% milling outturn which is average of varieties (BRRI, 2000).

Table 2. Consumption of fertilizer N, P and K in Bangladesh over the years since 1961

Year	Fertilizer consumption (000 tonnes)		
	N	P	K
1961	20.0	DNA	1.5
1970	99.2	34.0	10.4
1980	266.2	118.4	28.7
1990	609.2	231.8	90.0
2000	995.8	250.3	143.0
2010	1275.0	420.0	220.3

Source: IFA, 2011; DNA = data not available.

Table 3. N, P, K and S removal of four rice varieties

Rice variety	Grain yield (t/ha)	Total amount of nutrients (kg) removed by grain and straw per tonne of grain production				Reference
		N	P	K	S	
BR1	4.2	19.76	3.10	21.43	1.90	Choudhury *et al*., 1992
BR3	5.6	13.93	3.39	20.89	2.14	Choudhury *et al*., 1994c
BR11	5.2	17.69	3.08	22.31	1.92	Choudhury *et al*., 1994c
BR22	4.6	15.65	2.83	19.13	1.96	Choudhury *et al*., 1992

Sources and forms of nitrogen fertilizer. Now-a-days different sources and forms of N fertilizer are available in the market for commercial use. The most commonly used N fertilizer for rice crop is prilled urea (PU). Urea super granule (USG) is a modified form of urea having an average diameter of 11.5 mm. It has been developed at the International Fertilizer Development Centre (IFDC), United States of America. The superiority of USG over PU in rice culture has been found in many investigations (Roy, 1988; Craswell *et al*., 1985). Urea large granule (ULG), another modified form of urea having an average diameter of 7 mm, has been developed in the Netherlands. This modified form of urea, because of its larger granule size than PU, may go deep into the mud simply by force throwing, and thus may be expected to be more efficient than PU. Azollon, a slow release nitrogen fertilizer, has been developed in Germany. It is a urea-formaldehyde condensation product containing

Table 4. Fertilizer N uptake and recovery of MR185 rice in different soils as determined by the ^{15}N tracer technique

Soil series	Fertilizer N applied (kg/ha)	N uptake (kg/ha) by whole plant (grain and straw)		Fertilizer N recovery (%)
		Total N uptake	Fertilizer N uptake	
Guar	40	96.53	6.40	16
	80	133.21	18.49	23
	120	151.68	28.50	24
Hutan	40	54.39	8.02	20
	80	54.94	13.94	17
	120	47.66	14.22	12
Idris	60	67.82	23.74	40
	120	90.37	48.62	41
	180	116.75	74.44	41
Tebengau	60	53.31	23.72	40
	120	79.46	50.40	42
	180	93.02	68.63	38

Source: Choudhury (2000).

38% N. A field experiment was conducted on a clay loam soil at BRRI to evaluate the relative performances of PU, ULG, USG and azollon in wetland rice culture (Choudhury *et al*., 1994a). Considering grain yield, USG was significantly superior to PU and azollon; whereas, ULG had a slight edge over PU, but statistically not significantly different (Table 5). Total N uptake increased significantly in ULG and USG treated plots compared to the conventional PU treated plots. Agronomic efficiency and apparent recovery of added N were considerably higher with USG and ULG as compared to PU. Azollon was inferior to PU.

The increase in fertilizer N use efficiency due to the use of modified forms of urea will reduce environmental pollution problems like eutrophication (over enrichment in nutrients), production of greenhouse gases like nitrous oxide and nitrate toxicity in the ground water. However, these modified forms of urea are not commonly used by farmers. Farmers' awareness of environmental benefits of these practices should be created at farm level in order to use USG and ULG for rice production.

Methods of nitrogen fertilizer application. Generally, urea is applied as surface broadcasting which causes losses of urea by different mechanisms, and thereby N use efficiency becomes low. Sub-surface placement of N fertilizer into the anaerobic soil zone has been proposed by many investigators as a possible mean of increasing N use efficiency (Reddy and Mitra, 1985). Pneumatic urea injector, an instrument for deep placement of prilled urea, has been developed in the Netherlands. Prilled urea (PU) can be placed through injection by this instrument with necessary calibration into a depth of 8-10 cm. A field experiment was conducted on a clay loam soil at BRRI to evaluate the relative performance of PU surface broadcasting and PU injection for N use efficiency in wetland rice culture (Choudhury and Bhuiyan, 1994). In the surface broadcasting method, PU was applied in three equal splits (1/3 immediately after seedling establishment + 1/3 at active tillering stage + 1/3 at 5-7 days before panicle initiation); while in the injection method, PU was applied at a time immediately after seedling establishment. Three rates of N (29, 58 and 87 kg N/ha) were used in both methods of N application. A nitrogen control treatment was also used in the trial. The injection method gave higher grain yield over the surface broadcasting method at all the rates of applied N, however, the difference was significant only at 87 kg N/ha (Table 6). Straw yield and total N uptake were significantly higher with the injection method over the surface broadcasting method at all the rates of added N. Agronomic efficiency and apparent recovery of added N were considerably higher with the injection method.

Although deep place of PU is time consuming and laborious, this will reduce environmental problems by minimizing N fertilizer losses by volatilisation and denitrification in addition to increase in rice yield. Adoption of this technique at farm level by proper demonstration is necessary.

Nitrogen and sulphur interactions. Sulphur (S) is a secondary nutrient for all crops. The metabolism of N and S in plants is closely interrelated. As a result plant cannot utilize N properly in S deficient soils and conversely S utilization of plant is being adversely affected by N deficiency in soils (Shah *et al*., 1996). A field experiment was conducted on a silty clay soil at BRRI to study the synergistic effects of N and S on growth and yield of wetland rice (Choudhury *et al*., 1994b). A strong synergistic effect between N and S was observed (Table 7). At 0 kg N/ha, S application at 20 kg/ha increased grain yield by only 0.3 t/ha, while the same S rate increased grain yield by 0.9 t/ha at

Table 5. Effects of forms and sources of nitrogen fertilizer on grain yield of BR3 rice, total N uptake, agronomic efficiency and apparent recovery of added N

N rate (kg/ha)	N fertilizer form/source	Grain yield (tonnes/ha)	Total N uptake (kg/ha)	Agronomic efficiency (kg grain/kg added N)	Apparent recovery of added N (%)
0	-	2.9^{d}	36.7^{c}	-	-
87	Prilled urea	4.0^{b}	62.6^{c}	12.6	29.8
87	Urea large granule	4.4^{ab}	70.3^{b}	17.2	38.6
87	Urea super granule	4.6^{a}	91.0^{a}	19.5	62.4
87	Azollon	3.6^{c}	53.0^{d}	8.1	18.7

Values followed by different letters within a column are significantly different at 5% level by Duncan's Multiple Range Test (DMRT); source: Choudhury *et al*. (1994a).

Table 6. Effects of rates and methods of nitrogen fertilizer application on grain and straw yields of BR3 rice, total N uptake, agronomic efficiency and apparent recovery of added N

N rate (kg/ha)	Method of application*	Grain yield (t/ha)	Straw yield (t/ha)	Total N uptake (kg/ha)	Agronomic efficiency (kg grain/kg added N)	Apparent recovery of added N (%)
0	-	2.7	1.8	35.9	-	-
29	SB	3.3	2.0	44.3	20.7	29
58	SB	3.6	2.3	49.8	15.5	24
87	SB	4.0	2.6	55.9	14.9	23
29	I	3.7	2.5	51.4	34.5	53
58	I	4.0	2.8	59.2	22.4	40
87	I	4.6	3.4	69.9	21.8	39
LSD (0.05)		0.42	0.41	3.3	-	-

*SB = surface broadcasting; I = injection; source: Choudhury and Bhuiyan (1994).

Table 7. Effects of nitrogen and sulphur on grain yield of BR3 rice and agronomic efficiency of added N

N rate (kg/ha)	Sulphur rate (kg/ha)			Mean
	0	20	40	
Grain yield (tonnes/ha)				
0	2.9	3.2	3.2	3.1 c
60	4.0	4.1	4.2	4.1 b
120	4.1	5.0	5.2	4.8 a
Mean	3.7 b	4.1 a	4.2 a	
Agronomic efficiency of N[1]				
60	18.3	20.0	21.7	20.0
120	10.0	17.5	19.2	15.6
Mean	14.2	18.8	20.5	

Kg grain per kg added N compared to the plots those received neither N nor S; in a row/column, values followed by different letters are significantly different at 5% level by DMRT; source: Choudhury *et al*. (1994b).

120 kg N/ha. Similarly at 0 kg S/ha, N application at 120 kg/ha increased grain yield by 1.2 t/ha, while the same N rate increased grain yield by 1.8 t/ha at 20 kg S/ha. Agronomic efficiency of added N increased gradually with increasing S rates up to 40 kg S/ha.

In S deficient soils, combined application of N and S is necessary to optimize grain yield. Investigations in India showed that combined application of N and S increased N and S uptakes by rice significantly (Srivastava and Singh, 2007). This implies that interactions between N and S have large effects on N and S transfers to rice plants for increasing grain production. Generally farmers are not using S fertilizer for rice. Awareness should be grown at farm level for the benefit S fertilisation in S deficient soils for increasing N use efficiency in rice production.

Varietal difference in nitrogen response. The magnitude of N response may vary from variety to variety depending upon their agronomic traits like plant height and growth duration in addition to N fertility status of the soil. Therefore, variety and soil specific N fertilizer recommendation is necessary to get optimum yield (Saleque *et al*., 2004). Agronomic efficiency (kg grain/kg added N) varies among rice varieties (Table 8). Nitrogen fertilizer requirement for maximum grain yield varies among varieties (Choudhury *et al*., 2002). Nitrogen rate for the maximum yield of a rice variety can be estimated from the regression equation $Y = a + bx - cx^2$ as follows (Gomez and Gomez, 1984):

$$Ny = b/2c$$

where, Ny = N rate (kg/ha) for maximum yield. Determination of N rates for maximum grain yields of different varieties is necessary to avoid indiscriminate application of a single N rate for all the varieties. This information will help to reduce N fertilizer losses through indiscriminate application of N and thus reduces environmental pollution to some extent.

Nitrogen response of short and tall statured varieties. Nitrogen response may vary among rice varieties based on their plant stature in addition to growth duration. A field experiment was conducted using four rice varieties (BR1, BR3, BR14 and BRRIdhan 29) having different agronomic parameters (Table 9) in a clay soil at BRRI farm in 1993 (BRRI, 1996). BR1 and BR14 are short duration varieties, while BR3 and BRRIdhan 29 are long duration ones. Again heights of BR1 and BR3 were relatively shorter than BR14 and BRRIdhan 29. Number

Table 8. Grain yield of some modern rice varieties without and with fertilizer N

Rice variety	Grain yield (t/ha)			Agronomic efficiency**	Reference
	Without fertilizer N	With fertilizer N (120 kg N/ha)	Difference*		
BR1	2.60	4.70	2.10	17.50	Choudhury *et al*., 1997a
BR3	2.60	4.80	2.20	18.33	Choudhury *et al*., 1997a
BR14	3.00	5.20	2.20	18.33	Choudhury *et al*., 1997a
BR22	2.50	4.30	1.80	15.00	Choudhury *et al*., 1997b
BR25	3.10	5.10	2.00	16.67	Choudhury *et al*., 1997b
BRRIdhan 29	3.60	5.90	2.30	19.17	Choudhury *et al*., 1997a

* = differences were statistically significant at 5% probability level; ** = kg grain per kg added N.

of tiller as well as panicle production per unit area was the highest in BR1 followed by BRRIdhan 29, BR3 and the lowest in BR14. Six rates of N (0, 40, 80, 120, 160 and 200 kg N/ha) were used in the experiment. Grain yield response to added N varied among the varieties (Table 10). The most interesting finding is that the tall statured varieties (BR14 and BRRIdhan 29) out yielded the short statured ones (BR1 and BR3) in N control plots by 0.4 to 0.6 t/ha. This indicates that the tall statured varieties can exploit more soil N for grain production. Root mass density was relatively higher in tall statures varieties (BR14 and BRRIdhan 29) compared to short statured ones (BR1 and BR3) at 10-20 cm depth in N control plots (Table 11). This implies that having deeper root system the tall varieties were able to utilize more soil N for grain production compared to the short ones in N control plots which enabled them to produce extra grain without fertilizer input. So, modern rice varieties having relatively taller plant stature will be economically advantageous for marginal farmers to produce extra grain without fertilizer input.

Table 9. Some agronomic parameters of four modern rice varieties

Variety	Plant height (cm)	Growth duration (days)*	Tiller number/m^2	Panicle number/m^2
BR1	63	150	414	404
BR3	79	170	302	291
BR14	91	155	263	247
BRRIdhan 29	90	168	310	296

* = period started from date of nursery sowing; source: BRRI, (1996).

Table 10. Effect of N fertilisation on grain yield of four modern rice varieties

N rate (kg/ha)	Grain yield (t/ha)			
	BR1	BR3	BR14	BRRIdhan 29
0	$2.6^{d\,B}$	$2.6^{d\,B}$	$3.0^{d\,AB}$	$3.2^{c\,A}$
40	$3.4^{c\,BC}$	$3.2^{c\,C}$	$3.7^{c\,B}$	$4.7^{b\,A}$
80	$3.7^{c\,C}$	$4.2^{b\,BC}$	$4.3^{b\,B}$	$5.3^{a\,A}$
120	$4.7^{b\,B}$	$4.8^{a\,B}$	$5.2^{a\,AB}$	$5.4^{a\,A}$
160	$5.3^{a\,AB}$	$5.0^{a\,B}$	$5.3^{a\,A}$	$5.6^{a\,A}$
200	$5.1^{ab\,B}$	$5.2^{a\,AB}$	$5.2^{a\,AB}$	$5.7^{a\,A}$

Source: BRRI (1996); figures followed by a common letter within a column (small letter) or row (capital letter) are not significantly different at 5% level by Duncan's Multiple Range Test (DMRT).

Table 11. Effect of N fertilization on root mass density of four modern rice varieties at flowering stage

N rate (kg/ha)	Root mass density (mg/cm^3)				
	BR1	BR3	BR14	BRRI dhan 29	N Mean
	0-10 cm depth				
0	1.10	2.50	1.98	2.15	1.93^{d}
40	1.35	3.22	2.47	2.44	2.37^{cd}
80	1.56	3.35	3.02	2.61	2.64^{bc}
120	1.86	3.43	3.50	2.88	2.92^{ab}
160	2.05	3.49	3.54	3.07	3.04^{ab}
200	2.12	4.13	3.84	3.57	3.42^{a}
Variety mean	1.67^{B}	3.3^{A}	3.06^{A}	2.79^{A}	–
	10-20 cm depth				
0	0.06	0.08	0.10	0.16	0.10^{b}
40	0.07	0.15	0.17	0.18	0.14^{ab}
80	0.11	0.17	0.17	0.19	0.16^{a}
120	0.12	0.14	0.15	0.18	0.15^{ab}
160	0.04	0.13	0.13	0.15	0.11^{ab}
200	0.03	0.11	0.10	0.15	0.10^{b}
Variety mean	0.07^{B}	0.13^{AB}	0.14^{A}	0.17^{A}	–

Source: BRRI (1996); figures followed by a common letter within a column (small letter) or row (capital letter) are not significantly different at 5% level by DMRT.

***Nitrogen response of traditional and improved plant types*.** A field experiment was conducted on a clay soil at BRRI during 1994 to evaluate the N response behaviour of four rice varieties (NigerSail, BR22, Pajam and BR25) using six N rates (0, 30, 60, 90, 120 and 150 kg N/ha) in wetland culture (Choudhury *et al*., 1997b). The variety BR22 is the improved plant type of Niger-Sail while BR25 is the improved plant type of Pajam. Grain yield response to added N varied among the varieties (Table 12). While, NigerSail responded to added N up to 90 kg N/ha, its improved plant type BR22 responded up to 150 kg N/ha. Both Pajam and its improved plant type BR25 responded up to 120 kg N/ha. However, BR25 out yielded Pajam at all the rates of added N. Regression analysis indicated that the estimated response function between N rate and grain yield for all the varieties was quadratic in nature (Table 13).

Table 12. Effects of N fertilization on grain yield of four rice varieties

N rate (kg/ha)	Grain yield (t/ha)			
	NigerSail	BR22	Pajam	BR25
0	2.6	2.5	3.0	3.1
30	3.2	3.5	3.7	3.9
60	3.3	3.8	4.0	4.7
90	3.6	4.0	4.3	4.8
120	3.3	4.3	4.8	5.1
150	3.2	4.7	4.8	4.9

Source: Choudhury *et al.* (1997b).

Table 13. Regression equation and R^2 value relating grain yield and N rate for four rice varieties

Variety	Regression equation	R^2 value	Estimated N rate (kg/ha) for maximum yield
NigerSail	$y=2.681+0.018x–0.0001x^2$	0.86^*	90
BR22	$y=2.626+0.023x–0.0001x^2$	0.96^{**}	115
Pajam	$y=3.002+0.021x–0.0001x^2$	0.98^{**}	105
BR25	$y=3.119+0.032x–0.0001x^2$	0.98^{**}	160

* = significant at 10% level of probability; ** = significant at 1% level of probability; source: Choudhury *et al*. (1997b).

However, the rate of response as evidenced from the response co-efficient (b value) was higher in the improved plant types (BR22 and BR25) compared to their respective traditional plant types (NigerSail and Pajam). While, the b value was only 0.018 for NigerSail, it was 0.023 for its improved plant type BR22. Again the b value for Pajam was 0.021 against the b value of 0.032 for its improved plant type BR25. The b value is the slope of regression line which measures the estimated rate of response (either increase or decrease). This implies that improved plant type can utilize fertilizer N more efficiently for grain production compared to their respective traditional plant type. Estimated N rate for maximum yield varies among the varieties. Estimated N rates for maximum grain yield were 160, 115, 105 and 90 kg/ha for BR25, BR22, Pajam and NigerSail, respectively. This information implies that there are differences among rice varieties for N requirement for maximum grain production.

Conclusion

Nitrogen use efficiency in rice culture can be increased considerably by using modified forms of urea like USG and ULG. The efficiency of the conventional prilled urea in rice culture can be increased to some extent by injecting it into the sub-surface by the instrument "Pneumatic Urea Injector". In sulphur deficient soils, N use efficiency in rice culture can be increased by combined application of N and S. Modern rice varieties having relatively taller plant stature will be economically advantageous for marginal farmers to produce extra grain without fertilizer input. There are varietal differences for N requirement for maximum grain production. This information will be helpful to avoid indiscriminate application of a single N rate for all the varieties. These ways of increasing N use efficiency will reduce environmental pollution problems due to eutrophication (over enrichment in nutrients), production of greenhouse gases like nitrous oxide, and nitrate toxicity in the ground water.

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