

Rodents and the Restionaceae:
Implications of Plant-Herbivore Interactions
in the Fynbos.

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Abstract:

The determinants of grazing by the mouse, *Otomys irroratus* were examined in five species of Restionaceae in a fynbos ecosystem. This herbivore exerts a high grazing pressure on Restionaceae species, which is likely to have a considerable fitness cost to the plants. Seven plant characters were analyzed as possible determinants of this herbivory. These were: Nitrogen concentration, protein precipitating tannin, condensed tannin, water content, succulence, sclerophylly and diameter. Whereas the levels of these characters varied markedly among three parts of the culm, they varied only marginally between sexes of this dioecious plant, if at all. *Otomys* was highly selective of both the part and species of culm grazed. Grazing was deterred by high levels of sclerophylly, but was positively correlated with the concentration of protein precipitating tannin. However sclerophylly and tannin levels were negatively correlated indicating a trade-off between these two carbon-based defences. These results imply that carbon-based defences represents a considerable cost to the plant, and suggest herbivory to be a strong contending force in the selection for a high incidence of sclerophylly in the fynbos. Further implications of this interaction are discussed, as well as reasons for the effective deterrent of herbivory by sclerophylly, but not by tannin. The nitrogen contents of the five plants were similar and were not found to influence herbivore preference or defensive allocation. The nitrogen concentrations were however extremely low (less than 7mg.g^{-1}). It was surprising that these levels could support the growth of a mammalian herbivore, which suggests that low nitrogen concentration is not an effective deterrent of herbivory as proposed by Grubb (1992). Growth rates were somewhat varied but were not consistent with the predictions of the resource availability hypothesis (Coley *et al.*, 1985) at a local scale. The deterrent activity of sclerophylly may however represent an adaptation to defend the plant against the loss of nutrients to herbivores within the nutrient-poor fynbos biome.

Introduction:

Plants face attack from a wide variety of organisms, these include insect, mollusc and vertebrate herbivores, as well as bacterial and fungal infections, nematodes and other parasitic animals and plants (Levin, 1976; Baas, 1989; Swain, 1977). Consequently plants have evolved a number of means of defending themselves against such organisms. These may be: morphological adaptations e.g. spines (Grubb, 1992; Campbell, 1986; Milton, 1991), leaf shape and appearance (Brown and Lawton, 1991); chemical defences e.g. tannins, glycosides, terpenoids, steroids, and alkaloids (Whittaker and Feeny, 1971); or structural adaptations e.g. increased sclerophylly, fibre and lignification (Dafni, 1991; Belovsky and Schmitz, 1991). These defences are considered to be primarily effective against the ubiquitous forces of herbivory, which indicates herbivory to be the main driving force in the evolution and selection of such defences. Many of these adaptations may however serve other survival functions to the plant. For instance, they may increase leaf longevity, and enhance burning by contributing to the flammability and fuel load of a plant (Baas, 1989; Reich *et al.*, 1992).

Complexity of plant-herbivore interactions

The study of plant defence is bedeviled by complexity. Animals do not simply eat plants: specific types of animals eat certain parts of certain plants, and the parts and types of plant eaten may change with age of both the plant and the animal (Janzen,

1979; Freeland and Janzen, 1974). Furthermore plants allocate defences differentially to various organs and this defensive allocation varies markedly with season, growth form and age of the tissue (Bryant *et al.*, 1991; Glyphis and Puttick, 1988; Owen-Smith and Cooper, 1987). Also the cost to the plant, of losing an organ changes over the growth period of the organ (Harper, 1989). The understanding of energetic allocations are further complicated by compounds and structures which perform more than one function and by the metabolic costs of re-synthesising unstable compounds (Bazzaz *et al.*, 1987; Coley *et al.*, 1985). Lastly, only recently has the combined deterrent value of the interaction of the defensive characters within a plant been investigated (Cooper *et al.*, 1988; Hanley *et al.*, 1992).

The study of specific herbivore-plant interactions is preferable to broad-brush approaches at a landscape level since cause and effect relationships are more likely to be correctly inferred (Owen-Smith, 1993). Not only is feeding behaviour and preference peculiar to individual herbivores, but many defences are group or even species specific (Robbins *et al.*, 1991; Hanley *et al.*; 1992, Bryant *et al.*, 1991). For example: condensed tannins laid down on cell walls may be an effective defence against ruminants but are ineffective against sap-sucking insects (Zucker, 1983); duikers can tolerate high condensed tannin levels in their diet but many grazing ungulates cannot (Owen-Smith, 1993). It is thus important to identify and specify the dominant herbivore before identifying a particular plant trait as a defence.

Plant defence theories

Herbivory and defence has been a much researched field over the past 20 years. Yet it is not surprising that few hypotheses which adequately explain plants investment in defence and describe patterns or strategies of the latter, have been proposed. Many of the previously prominent hypotheses, for instance the apparency theory (Feeny, 1976), have little support today, and have been criticised as being too simplistic, and not reflecting the true complexity of the ecological interactions involved (Grubb, 1992).

Resource availability hypothesis

Coley et al. (1985) resource availability hypothesis is today, perhaps, the most widely accepted explanation of trends in plants relative investment in defence, and has been substantiated in a number of biomes, including: Arctic boreal forest (Bryant et al., 1992); lowland rainforest (Coley, 1988); and southern african savannas (Bryant et al., 1987; Owen-smith and Cooper 1987). This hypothesis suggests that plants adapted to resource-poor habitats (lacking nutrients or light) will be slow growing and will in turn invest more heavily in defence. Whereas plants with access to more resources will be fast growing and invest less in defence, since defoliation represents relatively less of a resource loss, and the effects of tissue loss can be mitigated through fast growth. The resource environment to which a plant is adapted underlies the evolution of intrinsic growth rates and defence allocation (Coley et al., 1985). Intrinsic growth rates are therefore an accurate determinant of relative defence allocation, since this evolved capacity is genetically fixed (Coley, 1988).

Sophisticated metabolic arguments contest the evolutionary basis for this relationship, but not the trend. The carbon/nutrient cycle hypothesis holds that in nutrient poor habitats where light is not limiting, plants are nitrogen limited. Thus plants are slow-growing because they lack the available nitrogen to turn photosynthate into growth. As a result, carbon is produced in excess by continued photosynthesis and this excess is channelled (via metabolic cycles) into carbon based defences. Thus the production of carbon based defences is not a cost but more of a means of disposing of excess photosynthate (Baas, 1989). The above two hypotheses have not been adequately differentiated, both indicate a similar trend, however the mechanism driving resource availability hypothesis is evolutionary and that driving the carbon/nutrient cycle is metabolic.

Grubb's (1992) hypothesis

Grubb (1992) proposed a scheme of defence allocation based on the relative availability of accessible nutritious material at the landscape level. He suggests that in the fynbos, where large-scale, even-aged stands arise after fire, the nutrient value of a plant relative to its neighbours is the dominating force in the evolution of plant defences. Nitrogen is considered to be the nutrient most deficient in the browse of herbivores, as a far higher proportion of animal tissue, than plant tissue consists of nitrogen (protein) and animals continuously lose nitrogen by excretion (Gulmon and Mooney, 1986). The majority of fynbos plants have extremely low nitrogen levels and these will not support the growth of most herbivores (Grubb, 1992). Grubb (1992) argues that mammalian herbivores in this environment must be highly selective feeders in order to survive. Indeed the antelope that occur in mountain fynbos (Skead, 1980),

fall within Jarman's (1974) classification of highly selective browsers. Thus the few plants with higher nitrogen concentrations stand out as islands of nutritious material in the landscape, and are hence at risk, thus these plants invest more heavily in defence. Grubb (1992) related this defence to the incidence of spinescence in fynbos plants but it applies equally to other defence characters.

Sexual dimorphism

Charlesworth and Morgan (1991) suggest that it may be incorrect to assume equal defence allocation in both sexes of dioecious species. Differences in nutritional quality, secondary compound concentration and grazing preference have been detected in some dioecious plants (Bryant *et al.*, 1991).

Female plants are generally assumed to make a greater reproductive investment, although Charlesworth and Morgan (1991) point out that the reproductive investment of male plants is high but of a short duration. It has been argued both that female plants will allocate less to defence as their reproductive investment is so high, and that they will allocate more to defence in order to protect their high reproductive investment (Charlesworth and Morgan, 1991).

Herbivory in the fynbos

Herbivores are presumed to be scarce in the fynbos. Certainly that is likely to be true of large mammalian herbivores. Even historic accounts, record few ungulates on mountain fynbos or the Cape Peninsula (Skead, 1980). Perhaps because herbivory is presumed to be rare, plant-herbivore interactions are relatively unstudied. Where

studies of herbivores have been done, they are largely restricted to lists of plants taken by specific herbivores (e.g. klipspringers; Norton, 1983). Although plant part and species preference may be indicated, the underlying determinants of this herbivory are rarely satisfactorily examined and the ecological and evolutionary implications for these plants have not been advanced.

Rodent species may however be common in fynbos vegetation (Bond *et al.*, 1980). *Otomys* sp. (Commonly *Otomys irroratus*) is locally abundant in reed beds (De Graaf, 1981). Vegetative material and grasses have been shown to be the preferred diet of this herbivorous rodent (Curtis and Perrin, 1979). In fact, it is likely that *Otomys* is responsible for the majority of mammalian herbivory in mountain fynbos (W. Bond, pers. comm.). In the western Cape, this mouse, locally, exerts a considerable grazing pressure on Restionaceae species. The nature of this grazing is likely to constitute a high reproductive and survivorship cost to these plants. Culms are severed (bitten off) just above ground level, thereafter various portions of the culm are eaten. Thus not only is much photosynthetic material destroyed but the terminal reproductive investment is lost (although not necessarily eaten). Unlike grasses, the meristematic region of Restioids is apical, thus grazed material is not easily replaced (Linder, 1991). This may have catalysed or modified the evolution of defensive characters in the Restionaceae.

A number of Restionaceae species may coexist within a single habitat. These plants are likely to have similar genetic potentials and share a common structure and growth form. The study of the *Otomys*-Restionaceae interaction thus presents an ideal

opportunity to test the relevance of the web of current defence hypotheses to the fynbos biome since much of the animal-plant complexity and environmental variability may be reduced. Furthermore, numerous studies have been conducted on the interaction of grazers and grassland communities (briefly reviewed by Vicari and Bazely, 1993) and on the implications of browsers for specific dicotyledonous shrubs and trees (briefly reviewed by Owen-Smith *et al.*, 1993). However studies on other monocotyledonous species and particularly selective browsing on monocots at a species level are lacking.

Aims of this study

In this study, I aim to determine the key characters determining selective herbivory of restioids by *Otomys*, and to interpret these in the light of current defence theory by examining the following questions:

Is defence allocation inversely related to growth rate?

Are plants with higher concentrations of nitrogen more highly defended?

Are there sexual disparities in allocation to defence, or other plant characters?

Is there a vertical or transverse variation in restioids allocation to certain defences, and how does this relate to herbivory?

Is there a trade-off between one kind of defence and another?

Materials and Methods:

Study site and species

A study site was chosen within the Silvermine Nature Reserve (34°05'45''S; 18°25'10''E), part of the Table Mountain chain on the Cape Peninsula (approx. 25km South of Cape Town). This site was chosen as it was identified as having a high incidence of *Otomys* herbivory and uniform restioid dominated vegetation. Sampling within a site largely eliminates variability due to environmental and biotic heterogeneity (e.g. species composition, population of herbivores and period of burn).

The study was conducted over a period of four months (mid-May to mid-September) during the winter of 1994, which is the rainy season in this mediterranean area. Restionaceae initiate new growth during this season (Linder, 1991), thus differences in the growth rate of actively growing plants could be measured.

Five restioid species were dominant and ubiquitous at the study site, and were used for further study. These were: *Staberoha cernua* (L.f.) Dur. & Schinz; *Thamnochortus lucens* (Poir) Linder; *Thamnochortus gracilis* Mast.; *Hypodiscus aristatus* (Thunb.) Krauss; *Willdenowia glomerata* (Thunb.) Linder.

Indices of herbivory

The average proportion of each of these restioid species grazed by *Otomys* was used as an index of herbivory. Although commonly known as the vlei-rat, *Otomys* belongs to the mouse family Cricetidae. This diurnal herbivore does not burrow but constructs well-defined runs in feeding areas (Skinner and Smithers, 1990; De Graaf, 1981). Grazing by *Otomys* is distinctive in that the mouse severs restioid culms at a 45° angle (W. Bond, pers. comm.).

This grazing behaviour allowed indices of herbivory to be determined, directly from the vegetation. In patches where there were a high number of clearly marked runs (and consequently a high incidence of herbivory), all target restioid species within 50cm of a run were examined. The percentage of culms severed by *Otomys* grazing (i.e. at 45°) was recorded, for each plant. Where the majority of culms had been grazed or otherwise removed, identifications were made from the roots and rhizomes. The diameter of each plant was measured. Sampling was replicated in ten different local sites and a total of between 33 and 50 plants were examined for each species.

Rodent trapping

In order to conduct controlled feeding trials and observe feeding behaviour of *Otomys*, baited traps were laid at the study site. Four lines of ten traps each, were laid. Each trap was placed ten paces apart and positioned primarily in the runs. Traps were checked every morning and evening for a period of three weeks. Unfortunately no

Otomys were successfully captured. Only five were caught over the three week period and all were found dead in the traps. I hope to conduct feeding trials at a later stage.

Restioid growth rates

The relative growth rates of the five target restioid species were measured over a 60 day period. Six, similarly sized plants of each species were selected. All selected plants grew within 40m of each other, although, for each species, plants were selected from as wide a range as possible within this area. Five newly emerged culms of each plant were marked with individual metal tags, and their heights recorded. Initial height measurements were made between the 27 and 30 June and final measurements, on the 31 August, hence the increment in the length of the culms was calculated. Growth rates were corrected to a uniform 60 days and expressed as a percentage of the average length of adult culms for each plant.

Restioid defence and nutritive characters

Sampling procedure

Seven characters were quantified for each species, namely: nitrogen, protein precipitating tannin concentration, condensed tannin concentration, water content, succulence, sclerophylly, and width. These characters were considered the most likely determinants of herbivore preference. For each character, the two sexes and three vegetative parts of the culm were quantified independently.

Culms were separated into:

- 1) **bottom** - bottom 1 or 2 internodes of the culm.
- 2) **bracts** - Sheathing bracts, which grow around the culm at the nodes, were removed from the culm and measured independently.
- 3) **top** - top 1 or 2 internodes of the culm.

All plants were harvested no longer than 36 hours before being analyzed. Green culms of the same age (current seasons reproductive culms) were harvested from similarly sized plants within the study site. For the chemical assays, all culms were harvested at midday from the North facing side of plants to minimise possible metabolic or carbon and nutrient balance differences due to increased insolation or daily rhythms. Once harvested, samples were immediately transported to the laboratory and placed in distilled water. Assays were only carried out once the culms had rehydrated.

Tannins

In order to identify the cellular localization of tannins, transverse sections were made of the various species of restioids. The modified Nitroso staining procedure of Stafford *et al.* (1987) was employed (see Appendix), since this staining reaction is specific for condensed tannins, forming a red to brown colour.

Since hydrolysable tannins are not known from monocot plants (Chesselet *et al.*, 1992; Swain, 1977), two chemical assays were considered appropriate for quantifying the concentration of tannins present in the plants. The radial diffusion assay for protein precipitating tannins is suitable for measuring the biological action of plant tannin in

binding to protein (Hagerman, 1987; Hagerman and Butler, 1989). The vanillin assay for condensed tannins is structure specific and indicates the quantity of condensed tannin (proanthocyanidins) present in a sample (Hagerman and Butler, 1989).

Culms were separated into the three parts as described above, for each sample a number of culms from a single plant were used. For the radial diffusion assay, a number of culms from four replicate plants were assayed for each part. Material for each sample was frozen in liquid nitrogen and manually crushed (for details of all extraction and assay methods, refer to the Appendix). After completion of the radial diffusion assay, the remaining material (previously frozen and ground) was dried in an oven at 30°C. Condensed tannin and total nitrogen assays were performed on this dried material. Three replicates were used for each plant part in these two assays.

The radial diffusion assay followed the method outlined by Hagerman (1987), except that 70% acetone and not 50% methanol was used as an extraction medium (see Appendix). A standard curve was constructed using tannic acid (Fig.1).

The vanillin assay for condensed tannins was carried out according to the revised method of Price *et al.* (1978). Catechin was used to determine the calibration curve (Fig.2)

Nitrogen

The total nitrogen content was determined by the Kjeldahl digestion process (Smith, 1980). Figure 3 represents the standard curve for the total nitrogen assay.

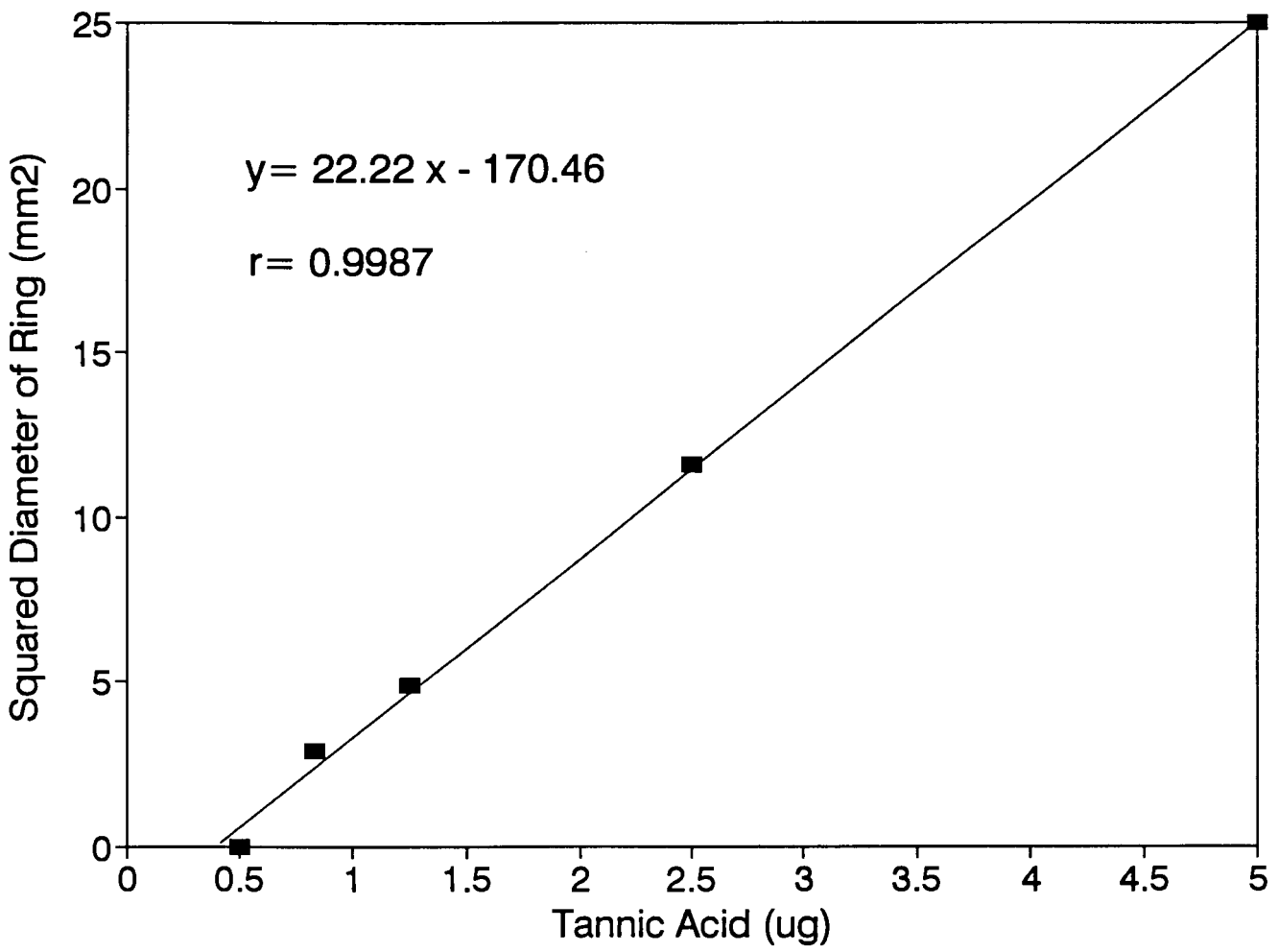


Figure 1. Standard curve for the radial diffusion assay for protein precipitating tannins, as determined with tannic acid. (d.f.=3)

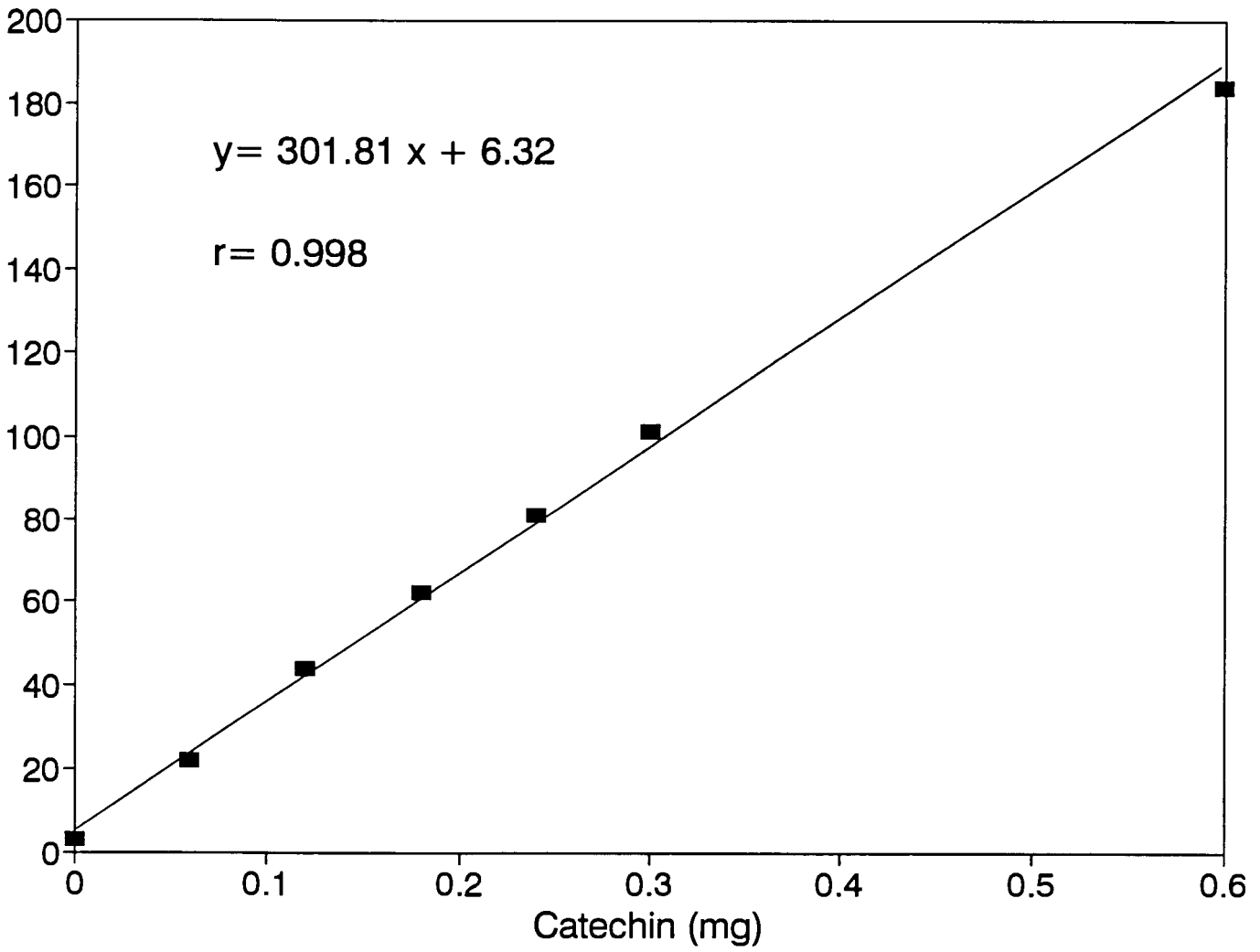


Figure 2. Standard curve for the vanillin assay for condensed tannins, as determined with catechin. Absorbance determined colorimetrically using a spectrophotometer. (d.f.=5)

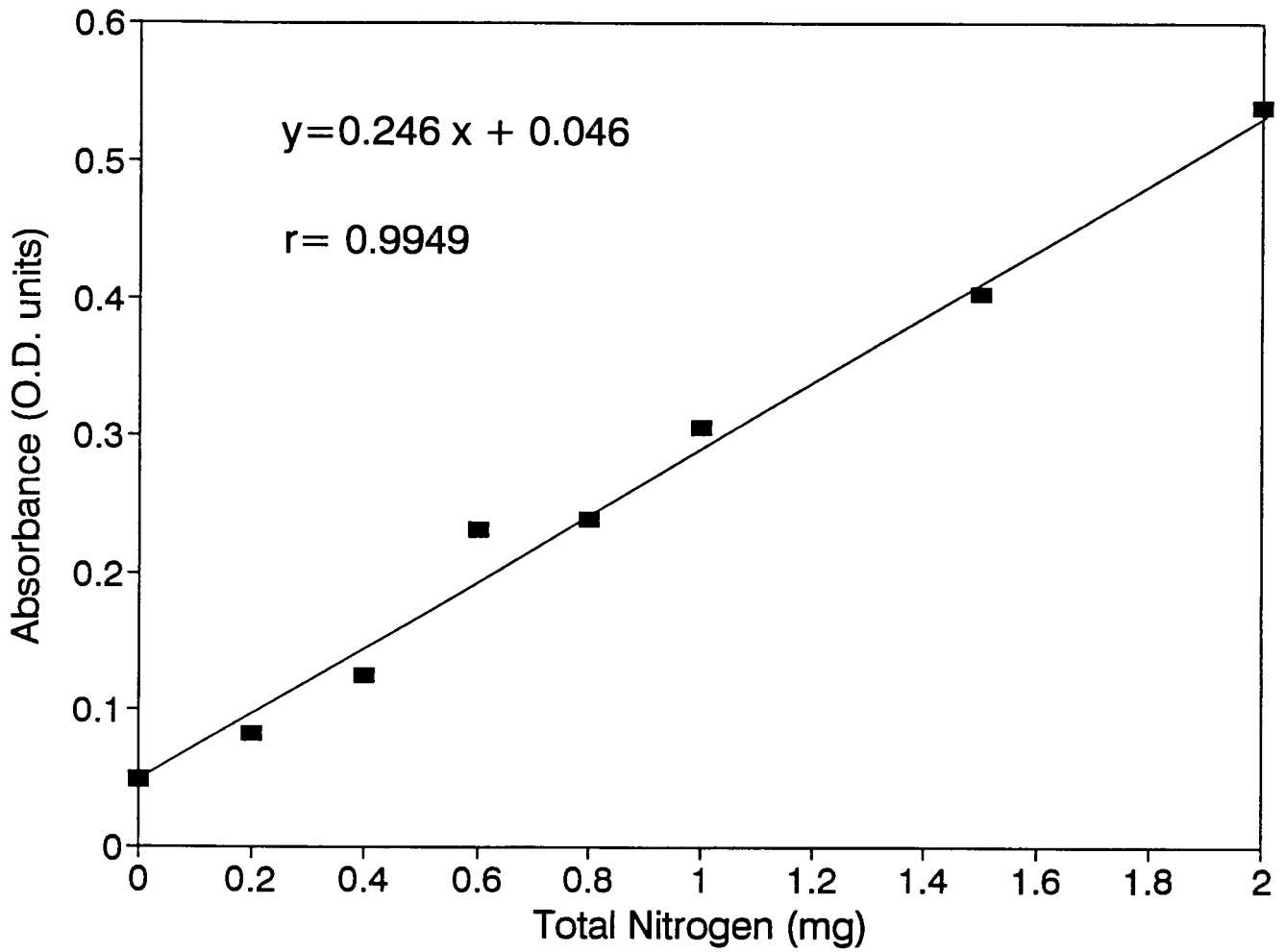


Figure 3. Standard curve for total nitrogen (calibrated with $(\text{NH}_4)_2\text{SO}_4$) as determined by the kjeldahl digestion process. Absorbance determined colorimetrically using a spectrophotometer. (d.f.= 6)

Culm diameter, water content, succulence and sclerophylly

Similarly, three replicate plants of the five restioids were rehydrated to maximum water content and then separated into parts. The cumulative length and average width of the restioid parts was determined for each replicate sample, using a leaf area meter. For each replicate, in the region of 40 bracts were measured, and about 20-30 internodes, for each culm top and culm bottom sample. All samples were then left in a convection oven at 30°C until dry.

The water content of each sample was calculated from the following formula, and the proportions expressed in percentages:

$$\text{(wet weight - dry weight)/dry weight}$$

The surface area of each part was calculated (as the surface area of a cylinder) as follows:

$$\text{average diameter} \times \pi \times \text{cumulative length}$$

Hence the degree of succulence and sclerophylly could be calculated according to the measures of Cowling and Campbell (1983):

succulence: **water content (g)/surface area (dm)**

sclerophylly: **dry weight (g)/surface area (dm)**

Statistical analyses

Chi-square analysis

To determine whether *Otomys* display selective preference in grazing or whether they simply consume restioids in proportion to their abundance in the environment, a chi-squared analysis was performed. The observed area of each species grazed was compared to the area that would be expected to be grazed if all species were simply consumed in proportion to their abundance. The total area covered by each species (available area) was calculated as the cumulative area covered by each plant. The area of each plant was calculated from the diameter of the plant, using the formula:

$$\text{area of plant} = \pi(\text{plant diameter}/2)^2$$

The observed area of each species grazed was determined by multiplying the proportion of each species grazed by the total available area. The expected area grazed was calculated by multiplying the available area of each species by the average proportion of all species grazed.

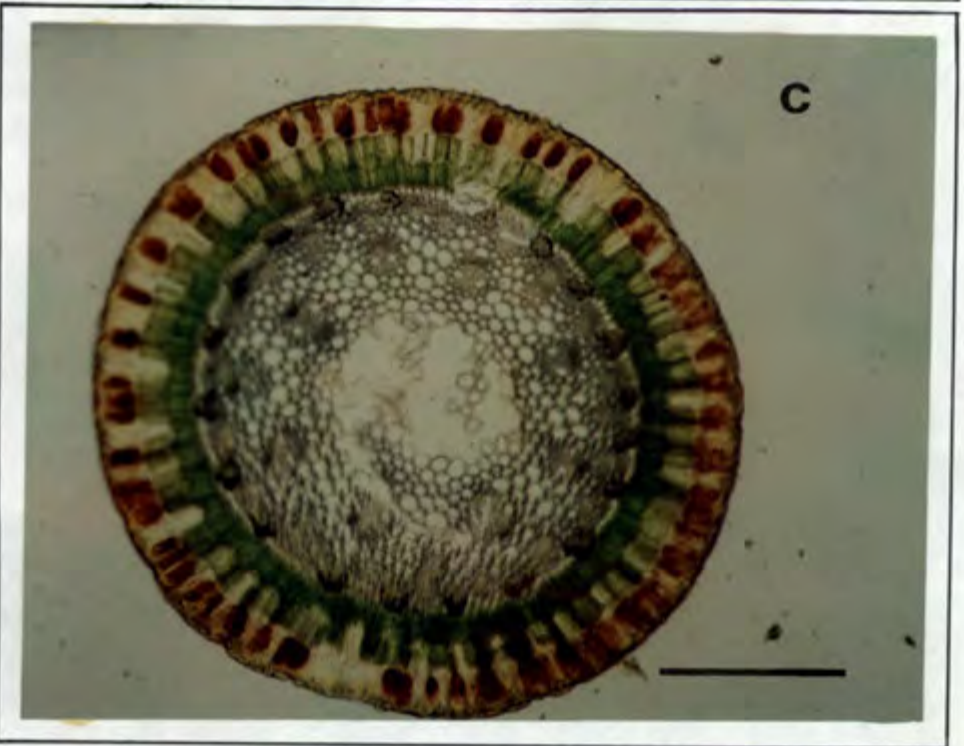
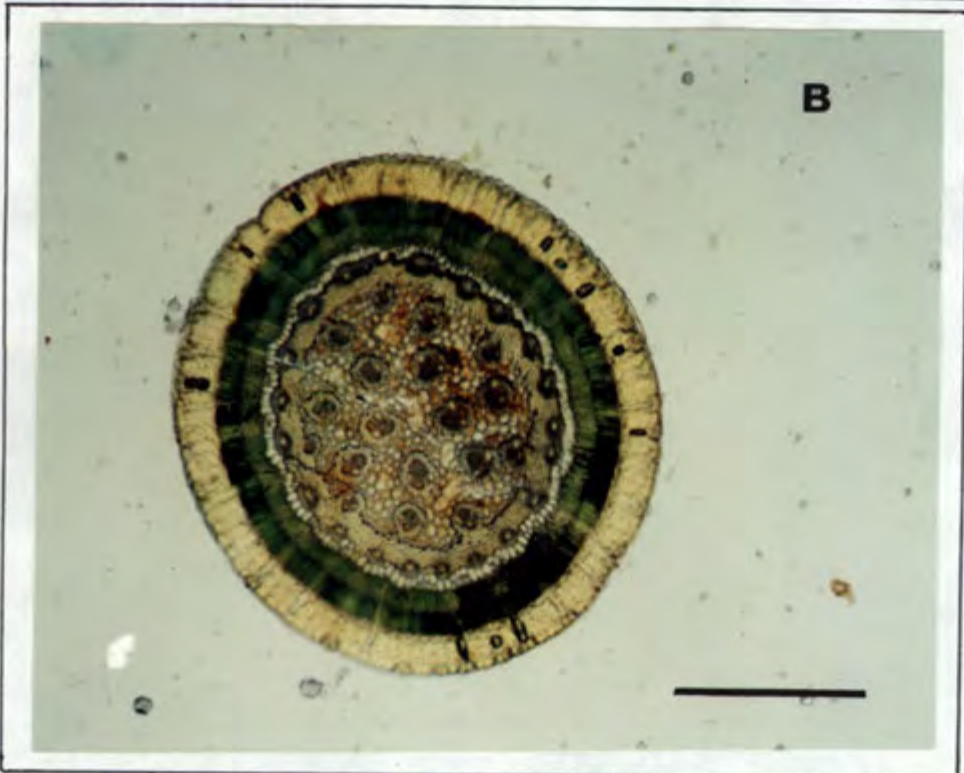
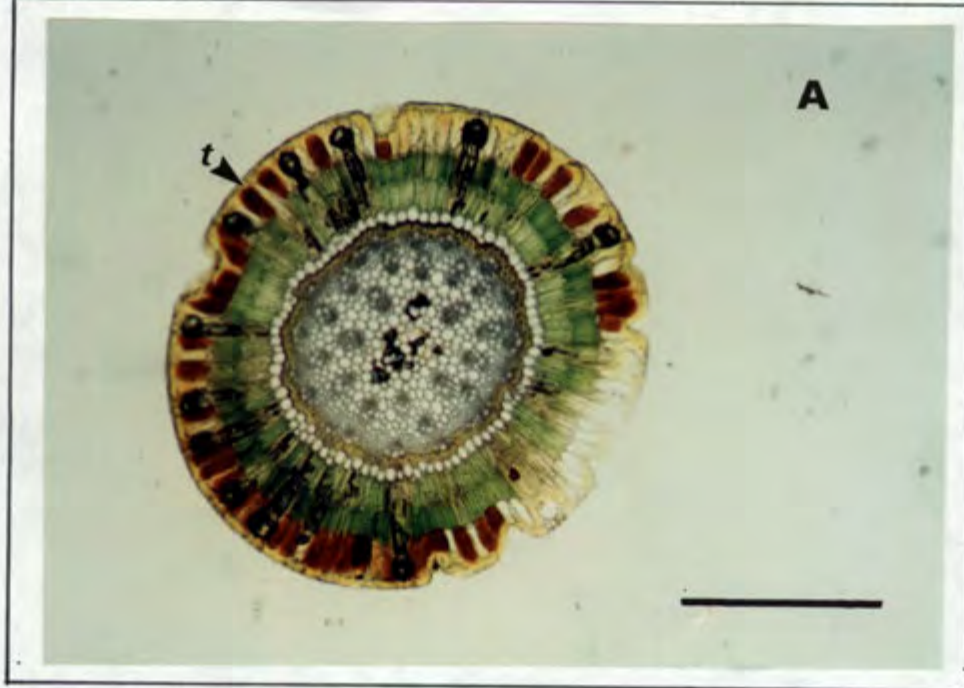
ANOVA

Three-way ANOVA's (species, sex, and part) were performed on each of the seven plant characters. One-way ANOVA's were performed on growth and feeding rate data. Significant differences between groups were determined by the Tukey multiple range test.

Much of the data for the various characters did not have a normal distribution. Although this violates the assumption of normality, Zar (1984) indicates that ANOVA is robust to this violation, particularly where there are a large number of observations (n) and when n is equal or nearly equal for all factors. Since the data are consistent with the latter conditions, ANOVA is likely to adequately indicate significant differences and trends in the data. Furthermore, the non-parametric, Kruskal-Wallis test was performed on all levels of the data and found to be consistent with the ANOVA.

Correlations

In order to perform correlations between growth rate and grazing preference, and each of the seven characters, average species values for the seven plant characters had to be calculated. These character values were calculated by taking an average of male and female, and culm top and bottom values (values for the bracts were not used since they are unlikely to influence herbivory of the entire culm). Every plant character was then also correlated against every other character.



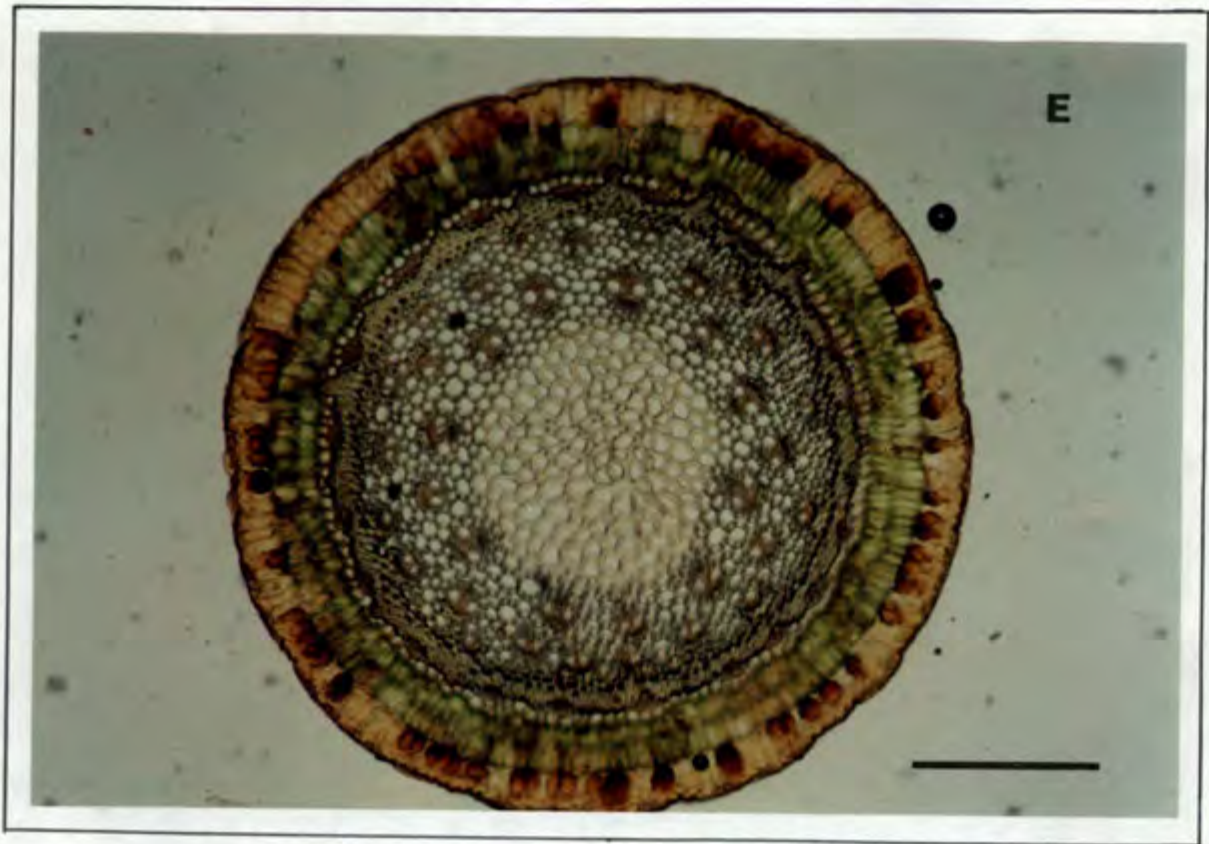
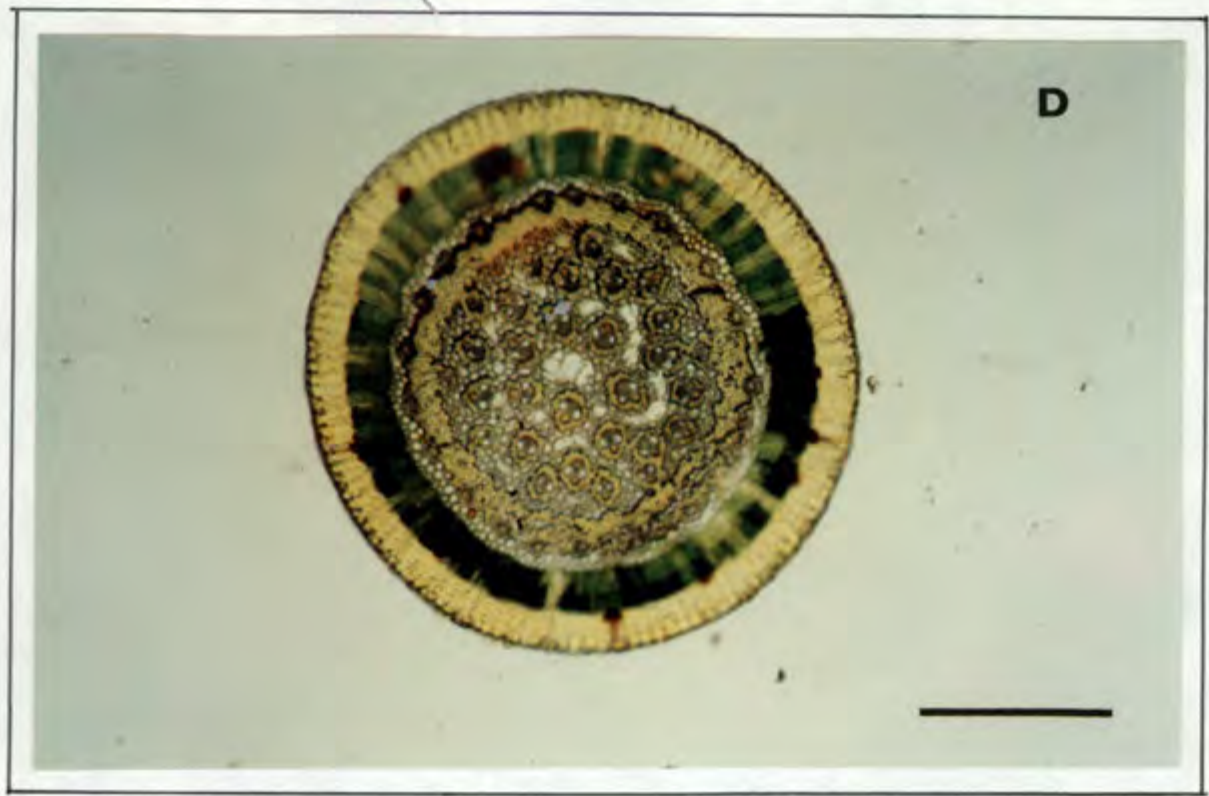


Plate 1. Transverse section through the culms of five species of Restionaceae, showing the tanniniferous cells in the epidermis (t). (A) *Staberoha cernua*; (B) *Thamnochortus lucens*; (C) *Hypodiscus aristatus*; (D) *Thamnochortus gracilis*; (E) *Willdenowia glomerata*. From the periphery, successive tissue layers are: epidermis; chlorenchyma; parenchyma; lignified sclerenchyma; pith containing vascular bundles. Scale bar = 500 μ m

Results:

Indices of herbivory

By comparing the observed and the expected area grazed for each restioid (Table 1), it is clear that these species are not simply grazed in proportion to their availability, and that *Otomys* is a highly selective grazer.

S. cernua appeared to be greatly favoured by *Otomys*, as it suffered markedly higher rates of herbivory than the other species, an average of 85% of culms being grazed (Table 2). Between 26% (*T. gracilis*) and 48% (*H. aristatus*) of the other species were grazed by *Otomys*. The ANOVA separated these restioids into three homogenous groups, but *T. lucens* and *W. glomerata* were not significantly different from the last two.

Table 1. Total area covered by each of the five studied Restionaceae species within 0.5m of ten *Otomys* runs, at Silvermine, Cape Peninsula. The area of each, grazed by *Otomys*, and the expected area grazed (calculated from the available area assuming equal grazing preference for each species).

Species	Available area (cm ²)	Observed area grazed (cm ²)	Expected area grazed (cm ²)	Chi-square statistic
<i>S. cernua</i>	2404	1929	845	1390.6
<i>T. lucens</i>	3926	689	1380	346.0
<i>H. aristatus</i>	14294	4190	5026	139.1
<i>T. gracilis</i>	6367	2506	2239	31.8
<i>W. glomerata</i>	4359	1709	1533	20.2
Total	31350	11023	11023	1927.7***

*** Highly significant $P \approx 0$

Table 2. Average proportion of each plant grazed by *Otomys*. The average growth rate of culms of each species, over a 60 day period, as a proportion of the average height of each plant. (± 1 standard error). Asterisks vertically aligned represent significantly similar groups.

Species	Ave. proportion grazed	Growth rate
S. cernua	84.82% (± 3.22) *	11.70% (± 0.89) **
T. lucens	34.20% (± 4.25) **	15.61% (± 2.00) *
H. aristatus	47.66% (± 4.20) *	15.02% (± 1.02) *
T. gracilis	25.54% (± 4.79) *	6.86% (± 0.83) *
W. glomerata	39.46% (± 3.46) **	16.33% (± 2.12) *

Other measures of herbivore preference did not differ dramatically from this index and did not provide any additional insights into possible determinants of herbivory. The foraging behaviour of *Otomys* has not been examined and appears to be little understood. Expressing the proportion of each restioid grazed relative to the availability of each, a measure which is consistent with the predictions of optimal foraging theory (Begon *et al.*, 1990), did contribute further to an understanding of determinants of foraging or herbivory. These herbivores are highly territorial (De Graaf, 1981) and are likely to forage in close proximity to their runs (pers. obs.), passing the same plants repeatedly on a daily basis. Thus the relative abundance of a particular species may be of little importance, provided it occurs in close proximity to a frequently used run.

Rodent trapping

A number of rodent species were trapped at the site including: *Rhabdomys pumilio*, *Acomys subspinosus*, *Mus minutoides*, *Mastomys verreauxii*, *Otomys* sp. The common *Otomys irroratus* is likely to be the dominant species of this genus, although *O. saundersiae* and *O. unisulcatus* have been reported from the Cape Peninsula. Positive identification can only be made by careful examination of the teeth. *Otomys* are the only fynbos rodents that feed entirely on green vegetative material. The other rodents are largely granivorous and supplement their diet with insects and vegetative material (Perrin and Curtis, 1979; Skinner and Smithers, 1990; De Graaf, 1981).

Growth rate

The growth rates of all the restioid species were similar except for *T. gracilis* which was significantly lower (Table 2). The other four species were not significantly different and apart from *S. cernua*, had growth rates of between 15.0% and 16.3%.

Restioid defence and nutritive characters

Nitrogen

Nitrogen concentration differed markedly between plant parts (Fig.4). In all species, nitrogen contents at the top of the culm were significantly higher than at the bottom, the contents of tops being approximately 8mg.g⁻¹, peaking at 9.1mg.g⁻¹ in male *Staberoha cernua*. Nitrogen levels of culm bottoms ranged from 3.2mg.g⁻¹ (female

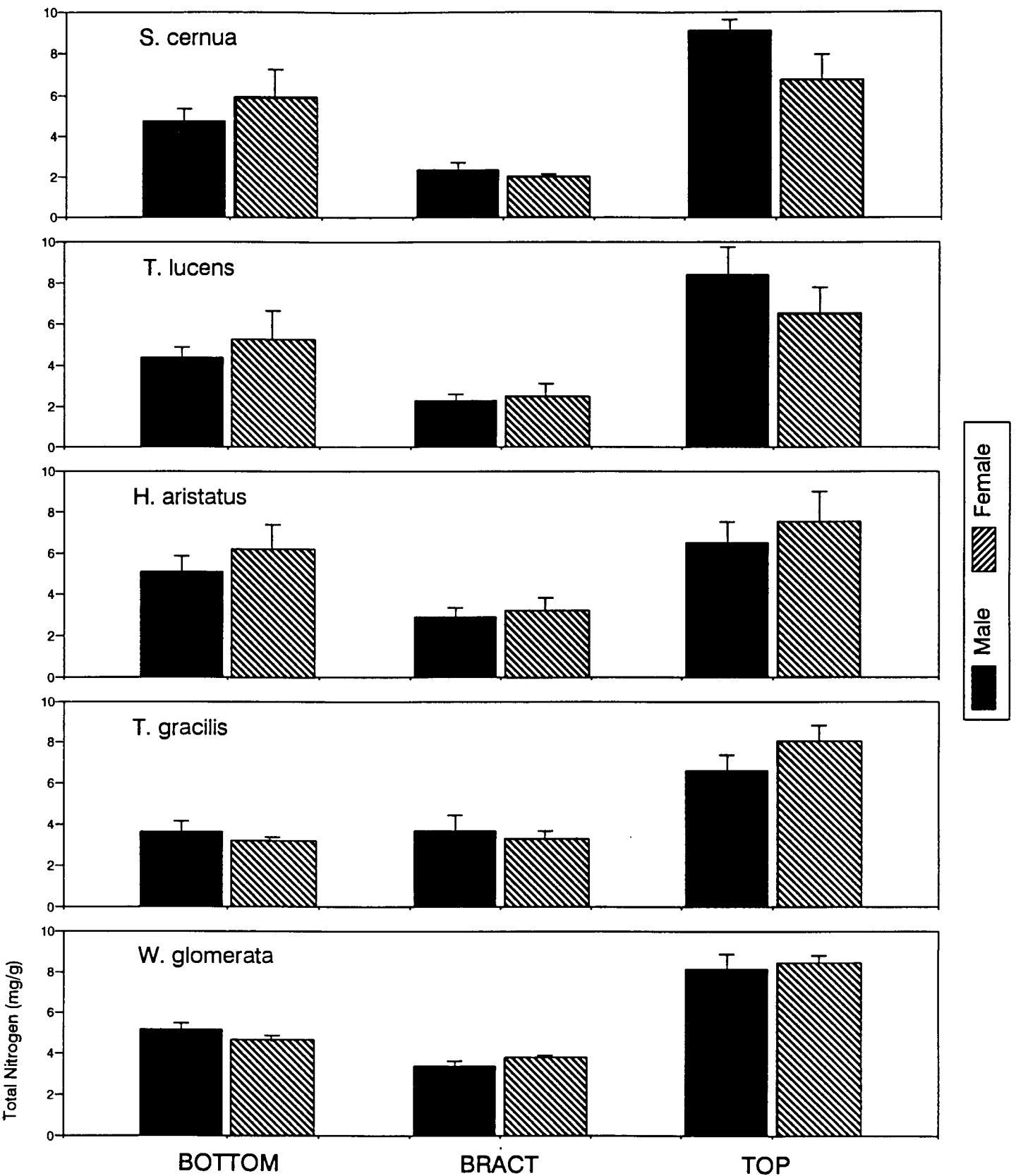


Figure 4. Nitrogen concentration of five Restionaceae species from the study site at Silvermine, Cape Peninsula. Values are given for males and females and three plant parts for each species. BOTTOM=bottom 1 or 2 internodes of the culm; TOP=top 1 or 2 internodes of the culm; BRACTS=shathing bracts around the culm. For each bar, n=3. Vertical lines=1 Standard Error.

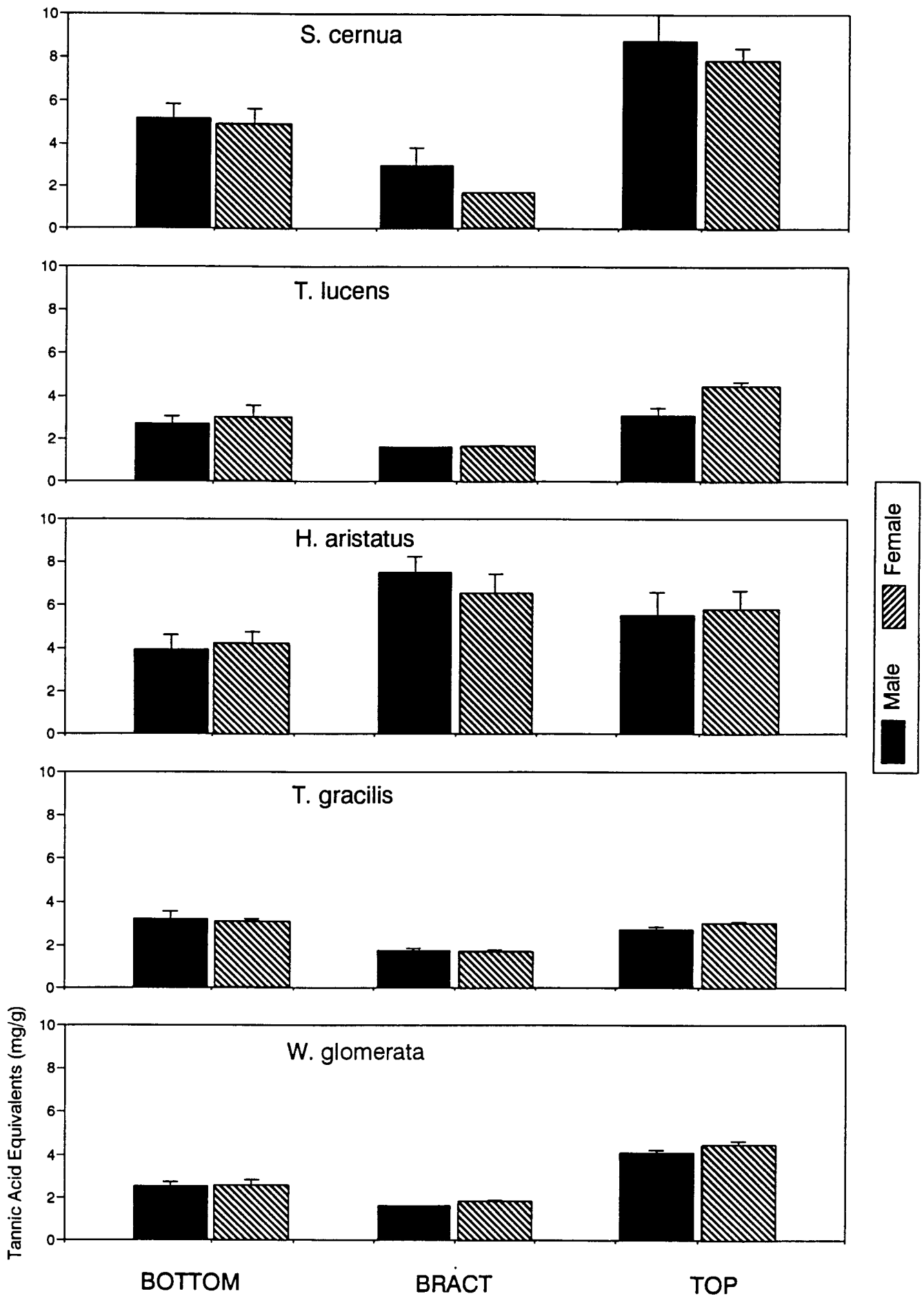


Figure 5. Protein precipitating tannin concentration of both sexes, and three vegetative parts of five Restionaceae species. Values were determined by the radial diffusion assay and expressed in mg tannic acid equivalents per g fresh weight. BOTTOM=bottom 1 or 2 internodes; TOP=top 1 or 2 internodes; BRÄCTS=shathing bracts. For each bar, n=4. Vertical lines=1 Standard Error.

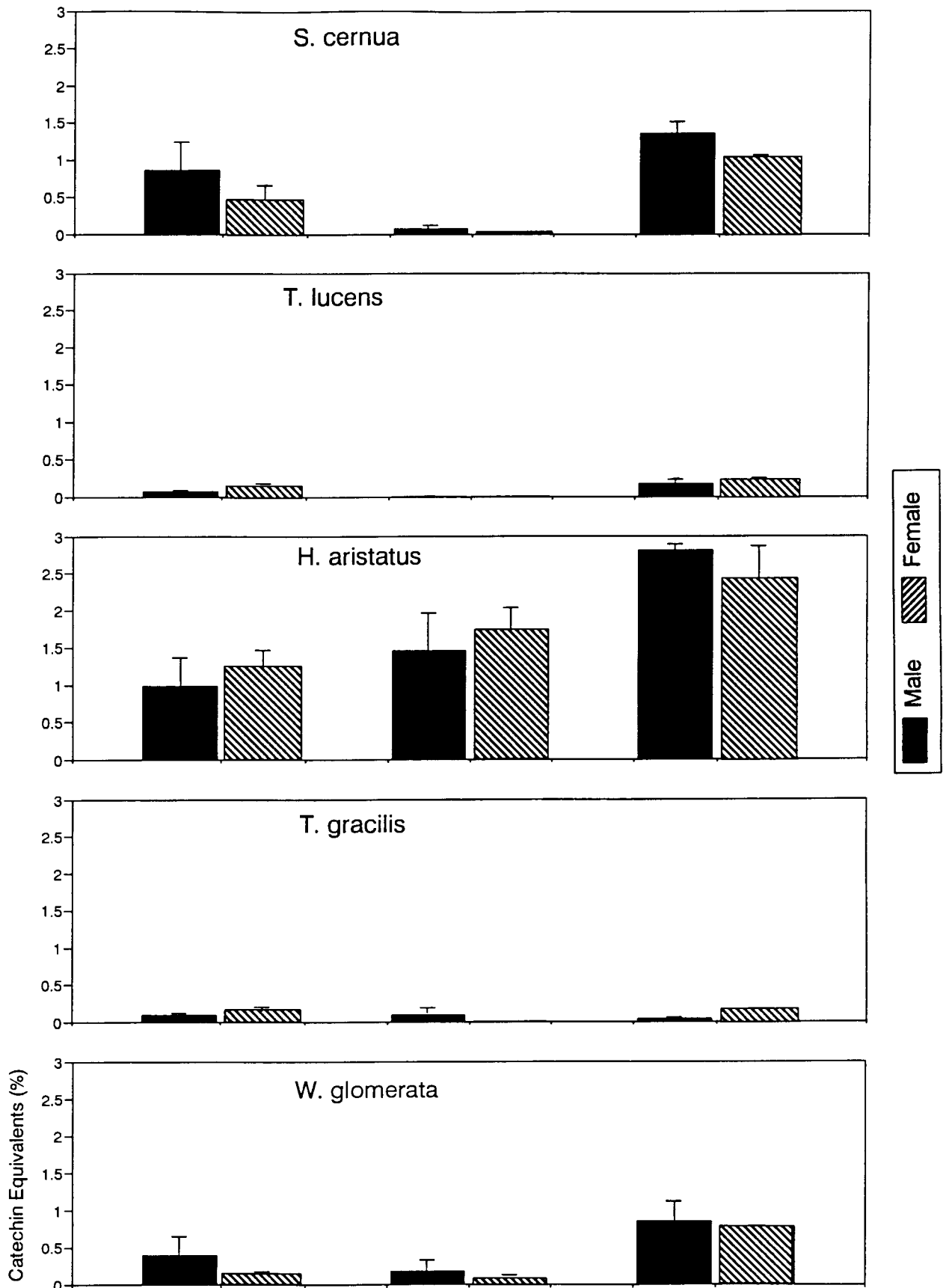


Figure 6. Condensed tannin concentration of both sexes, and three vegetative parts of five Restionaceae species. Values were determined by the vanillin assay and expressed in mg catechin equivalents per g dry weight. BOTTOM=bottom 1 or 2 internodes; TOP=top 1 or 2 internodes; BRACTS=sheathing bracts. For each bar, n=3. Vertical lines=1 Standard Error.

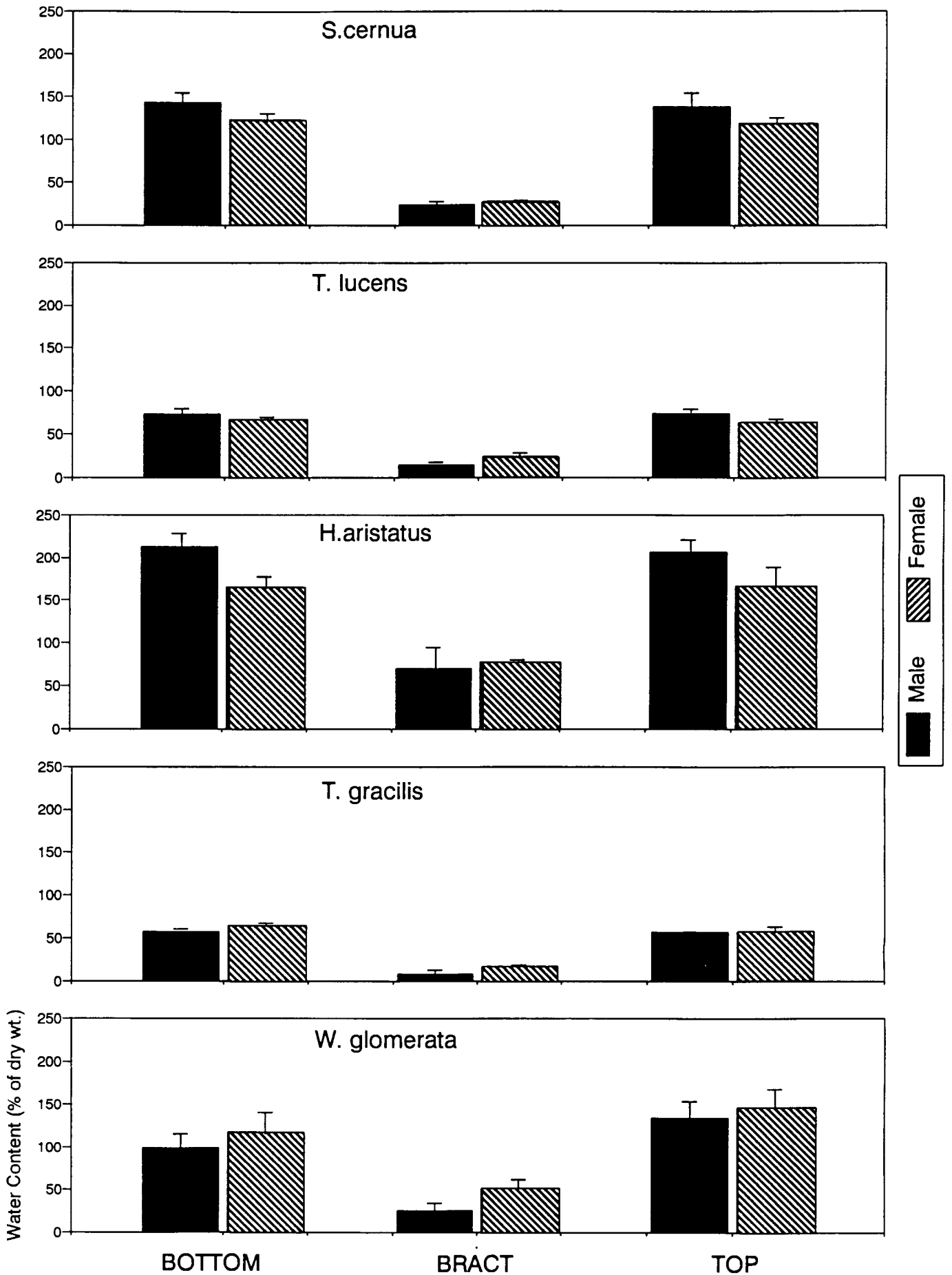


Figure 7. Water content (maximal) of five Restionaceae species. Values for both sexes and for three vegetative parts are given. BOTTOM=bottom 1 or 2 internodes; TOP=top 1 or 2 internodes; BRACTS=shathing bracts. For each bar, n=3. Vertical lines=1 Standard Error.

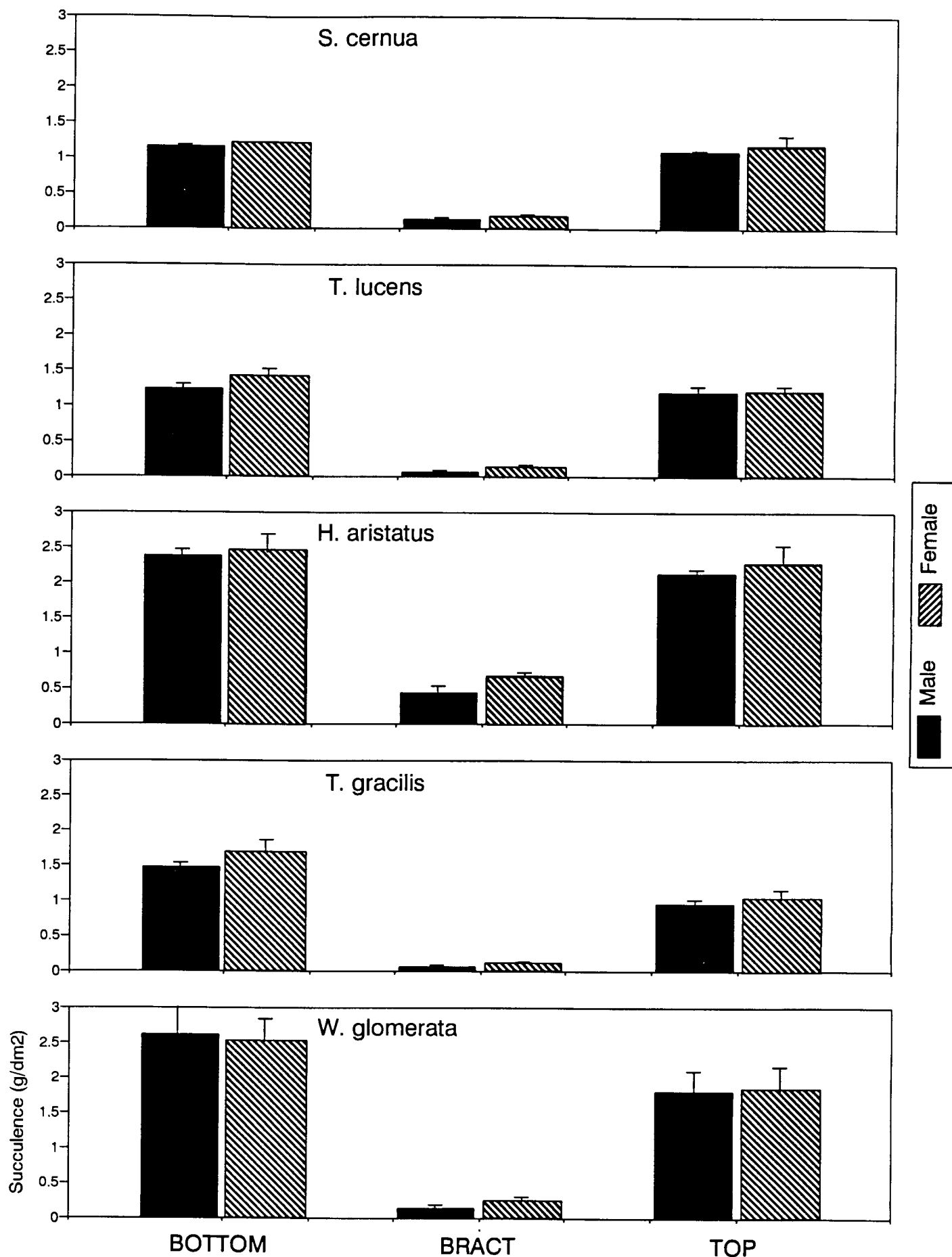


Figure 8. Succulence (g maximal water content per dm² culm surface area) of five Restionaceae species. Values for both sexes and for three vegetative parts are given. BOTTOM=bottom 1 or 2 internodes; TOP=top 1 or 2 internodes; BRACKTS=sheathing bracts. For each bar, n=3. Vertical lines=1 Standard Error.

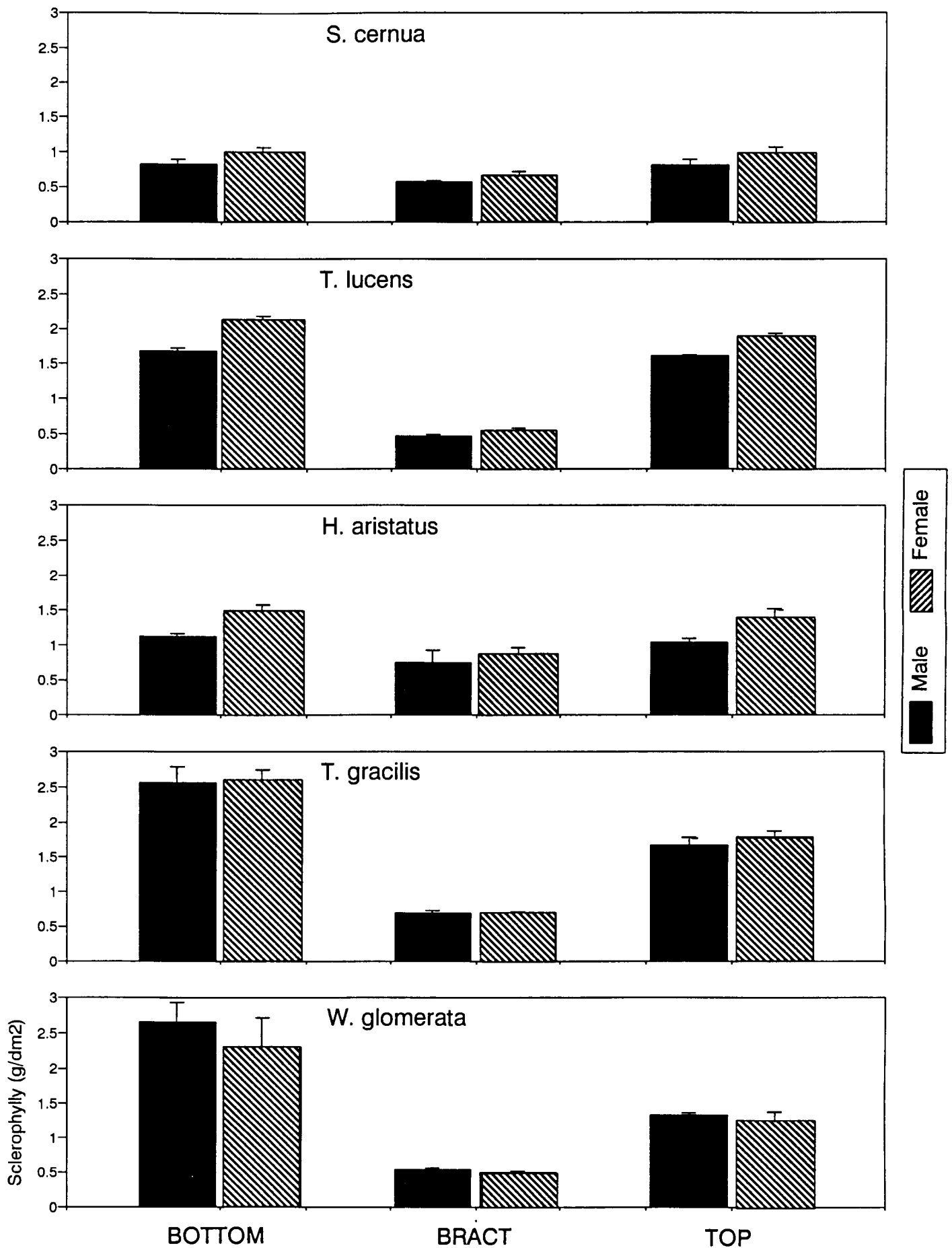


Figure 9. Sclerophylly (g dry weight per dm² surface area) of five Restionaceae species. Values for both sexes and for three vegetative parts are given. BOTTOM=bottom 1 or 2 internodes; TOP=top 1 or 2 internodes; BRACKTS=sheathing bracts. For each bar, n=3. Vertical lines=1 Standard Error.

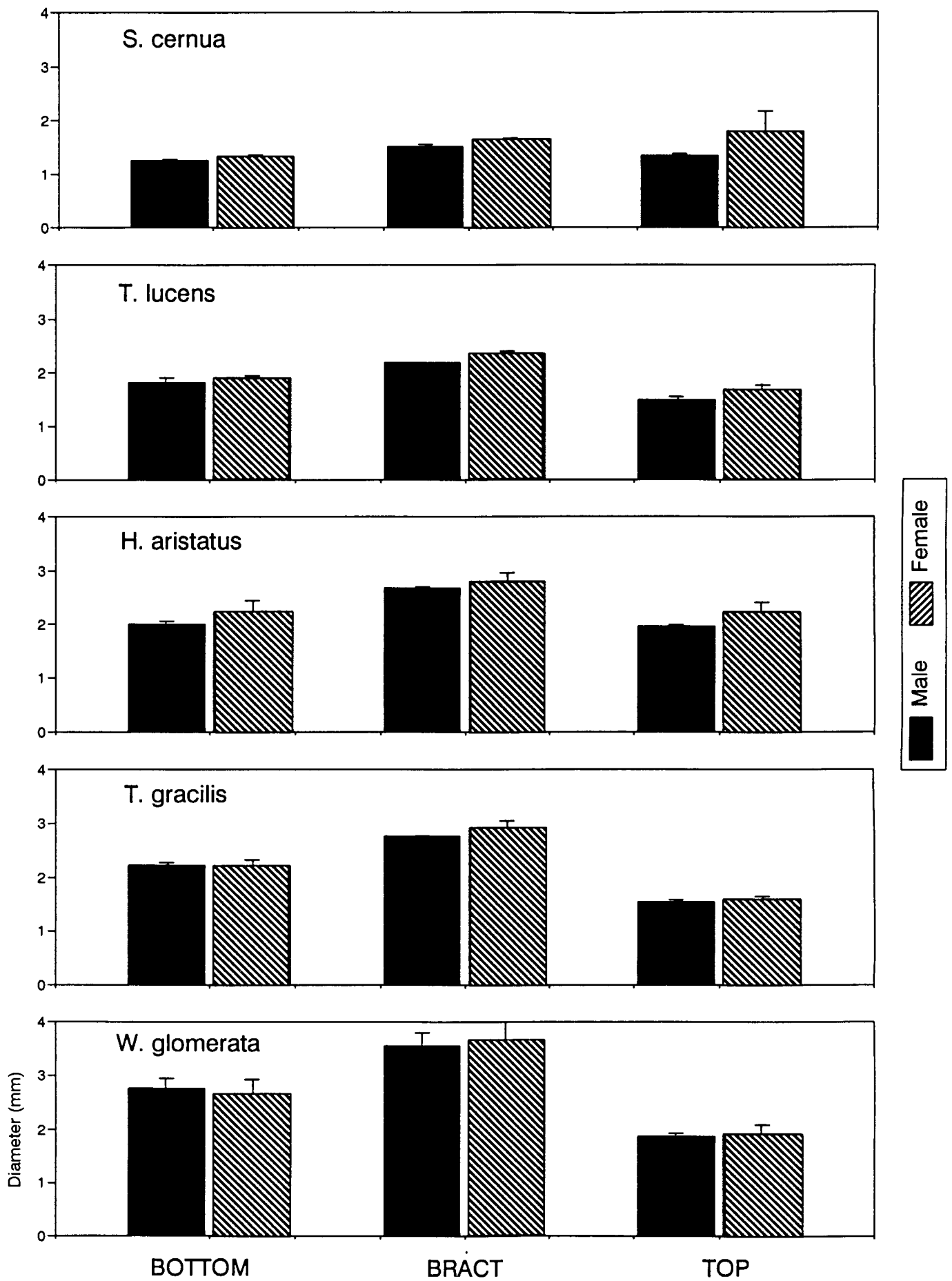


Figure 10. Culm top, culm bottom and bract diameter of both sexes of five Restionaceae species. BOTTOM=bottom 1 or 2 internodes; TOP=top 1 or 2 internodes; BRACTS=sheathing bracts. For each bar, n=3. Vertical lines=1 Standard Error.

Table 3. Results of the three-way ANOVA for each of seven plant characters. Asterisks vertically aligned represent significantly similar groups. **NIT**=nitrogen content; **PTAN**=protein precipitating tannins; **VTAN**=condensed tannins; **W.C.**=water content; **SUCC**=succulence; **SCL**=sclerophylly; **WIDTH**=diameter

	NIT.	PTAN	VTAN	W.C.	SUCC	SCL.	WIDTH
Species							
S. cernua	*	*	*	*	*	*	*
T. lucens	*	*	*	*	*	*	*
H. aristatus	*	*	*	*	*	*	*
T. gracilis	*	*	*	*	*	*	*
W. glomerata	*	*	**	*	*	*	*
Part							
bottom of culm	*	*	*	*	*	*	*
bract	*	*	*	*	*	*	*
top of culm	*	*	*	*	*	*	*
Sex							
male	*	*	*	*	*	*	*
female	*	*	*	*	*	*	*
Significant Interaction							
Species X Part	No	Yes	Yes	Yes	Yes	Yes	Yes
Species X Sex	No	No	No	Yes	No	Yes	No
Part X Sex	No	No	No	No	No	No	No
Species X Part X Sex	No	No	Yes	No	No	No	No

Thamnochortus gracilis) to $6.2\text{mg}\cdot\text{g}^{-1}$ (female *Hypodiscus aristatus*), and the levels of bracts were the lowest, in the region of $2\text{-}3\text{mg}\cdot\text{g}^{-1}$. This pattern was consistent across all species. Despite the variation among plant parts, the levels of the various species were remarkably similar and none were found to differ significantly (Table 3). No significant difference was recorded between the sexes of these restioids.

Tannins

In transverse section, the distribution of tannin is localised entirely within the epidermis (Plate 1).

The values for protein precipitating tannins showed interspecific variation (Fig.5, Table 3), the concentration of *Willdenowia glomerata*, *Thamnochortus lucens* and *T. gracilis* being low and that of *S. cernua* and *H. aristatus*, higher. The overall tannin concentration was however low, with no part containing more than $10\text{mg}\cdot\text{g}^{-1}$ (tannic acid equivalents), the highest was that of *S.cernua* tops at $8.8\text{mg}\cdot\text{g}^{-1}$. Generally culm tops had the highest tannin concentrations, and bracts the lowest, except in *H. aristatus* where bracts had the highest concentrations, which led to a significant interactive effect between species and culm parts (Table 3).

The radial diffusion assay was not sensitive to very low tannin concentrations, thus the standard curve has a non-zero intercept (Fig.1). Consequently, the samples were not found to have tannin concentrations of less than $1.6\text{mg}\cdot\text{g}^{-1}$ (tannic acid equivalents), when absorbences were read off from the standard curve. This may be a slight over-

estimation, the tannin levels of the bracts likely to be closer to zero as suggested by the vanillin assay (Fig.6).

The overall values for condensed tannins were somewhat different. *H. aristatus* had significantly higher values than the other species (Fig.6, Table 3). The values of *T. lucens* and *T. gracilis* were far lower than those of the other species, relative to protein precipitating tannins. The condensed tannin values for these two species ranged from 0.06% (catechin equivalents) for bracts to 0.22% for *T. lucens* culm tops. *S. cernua* had intermediate tannin contents but *W. glomerata* values were not significantly different from (low) *Thamnochortus* or the (moderate) *S. cernua* values. The pattern of values for restioid species and parts were similar to those of protein precipitating tannins except that *H. aristatus* tops had the highest values. Bracts however, generally had higher values than bottoms. This is likely to account for the significant species-part interaction (Table 3). Unlike Protein precipitating tannins, culm bottoms were not significantly different from bracts. The significant interaction effect of species, part and sex is unlikely to be biologically meaningful as sex differences were not found to be significant.

The vanillin assay is specific for condensed tannin (proanthocyanidin), however the reaction intensity is dependent of the chemical structure of compounds in the sample and vanillin does not react equally with all groups of condensed tannins (Hagerman and Butler, 1991). Hydrolysable tannins are unlikely to be present in these samples (Swain, 1977; Chesselet *et al.*, 1992), however the protein precipitating assay may be

sensitive to certain condensed tannins and tannin-like-substances outside the range of the vanillin assay. The results of the radial diffusion assay and the vanillin assay are not directly comparable, since compounds of vastly different chemical structures were used as standards, and the two assays were conducted on fresh and dried samples respectively. The protein precipitation assay is likely to be a more biologically important assay, since it measures the potential biological activity of a tannin in a sample (Hagerman, 1987), Whereas the vanillin assay is suitable for determining the quantity of a particular chemical structure present in a sample (Hagerman, 1989).

Water content, succulence

The water content of the restioid species varied considerably (Fig.7). *H. aristatus* had the highest water content, 165-213% (tops and bottoms), and *T. gracilis* contained the least water, around 57% (tops and bottoms). Culm tops and bottoms differed very little, although bracts had significantly less water (Table 3). The significant species-part interaction indicated that part differences do not vary in the same proportion among species, and the species-sex interaction is merely a result of neither sex having consistently higher values across all species. The trends for succulence values were the same as those of water contents (Fig.8), which is not surprising since the former is measure of water content per surface area, and the latter, water content per weight. The ANOVA however separates the species into two homogenous groups (Table 3), as opposed to three with water content. *W. glomerata* had as high succulence values as *H. aristatus*, and *S. cernua* had similar values to the two *Thamnochortus* species. Furthermore, culm bases were found to be significantly more succulent than tops.

Sclerophylly

Sclerophylly values for the restioid species were fairly evenly distributed (Fig.9), the ANOVA separated them into four homogenous groups (Table 3). *T. gracilis* was the most sclerophyllous, (bases, 2.6g.dm⁻²), *T. lucens* and *W. glomerata* had the next highest sclerophylly levels, followed by *H. aristatus* and finally *S.cernua* with the lowest value (all parts being less than 1g.dm⁻²). The three parts were significantly different, generally bases were more sclerophyllous than tops and bracts the least, but there was a significant species-part interaction. There was a significant difference in sclerophylly between male and female culms. Female culms were consistently more sclerophyllous except in *W. glomerata* where the trend was reversed, which gave rise to a significant sex-species interaction. The differences were however relatively small, males having an average value of 1.22g.dm⁻² and females 1.34 g.dm⁻².

Culm diameter

The diameter of female restioids was also significantly and consistently greater than males, although once again this difference was slight (Fig.10). Not surprisingly, bracts had the greatest diameter (since these grow around the culm), and bases were broader than tops. There were significant differences in the diameters of all but two species. *W. glomerata* had the highest average values of 2.74mm and *S.cernua* the lowest, at 1.48mm (Table 3).

Correlates of restioid defences

Growth rate was not significantly, inversely correlated with any defence character or with herbivory.

Nitrogen was not found to significantly influence herbivory, nor were any of the other plant characters or defences correlated with nitrogen levels.

The average rate of herbivory_{yr} correlated with the concentration of protein precipitating tannins (although not with condensed tannins), but this correlation was positive! Thus suggesting that plants with higher levels of tannins were more highly grazed. However sclerophylly was negatively related to grazing, and protein precipitating tannins correlated negatively with sclerophylly (Fig.11).

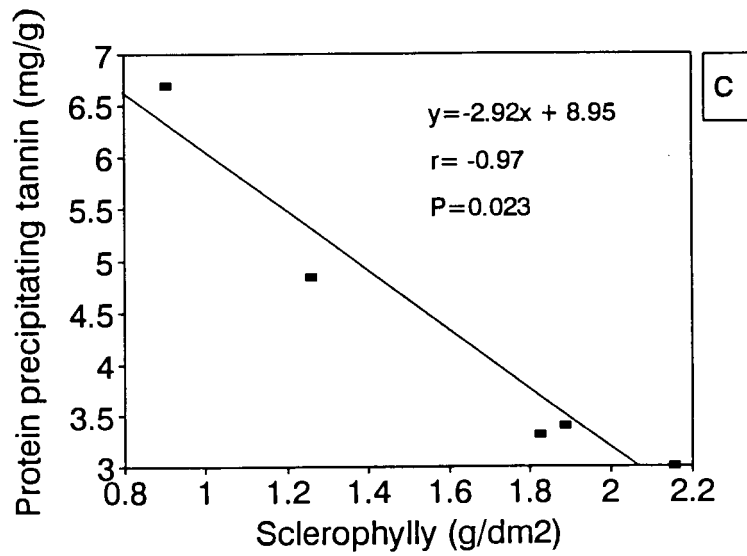
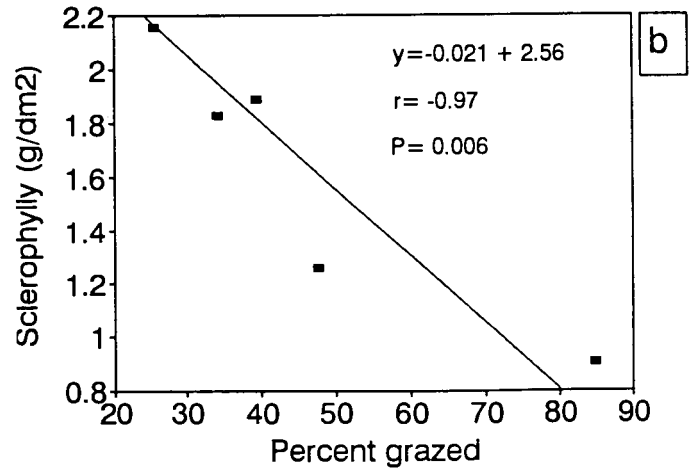
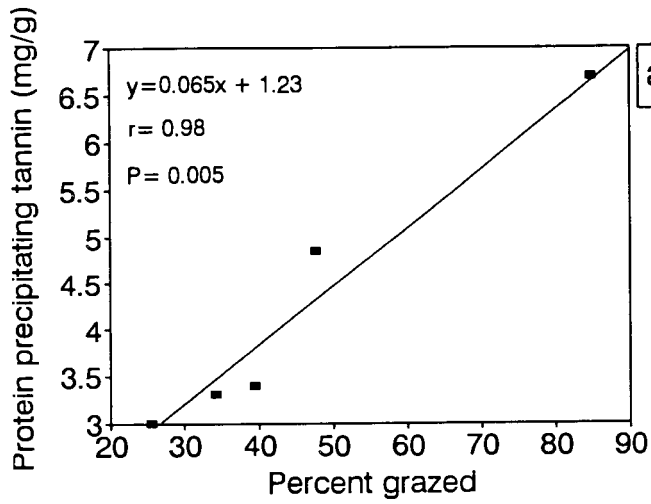


Figure 11. Regression analyses of two plant characters and an index of *Otomys* herbivory in five Restionaceae species. (a) concentration of protein precipitating tannin and herbivory index; (b) levels of sclerophylly and herbivore preference; (c) concentration of protein precipitating tannin and levels of sclerophylly.

Discussion:

Sclerophylly as a defence

High levels of sclerophylly are indicated to effectively deter herbivory (Fig.11). Sclerophylly (leaf specific weight is usually used as an index of sclerophylly, as in this study) is a measure of the fibre (lignin and cellulose) content of a plant organ (Stock *et al.*, 1992). Sclerophylly functions to reduce herbivory in two ways. Firstly, the increased lignin and cellulose contents dilute the nitrogen content of the culm, thus diminishing the nutritional quality of the organ (Stock *et al.*, 1992). Secondly, these fibrous tissues contribute greatly to the bulk of the forage but are largely indigestible or require specialised organs and long digestion times to be digested. This is perhaps best understood in the context of optimal foraging theory. Cellulose is fermentable in the caecum (and rumen) to yield digestible carbohydrates. However this requires a long digestion time and the rewards are small. Lignin is not fermentable but is time and energy consuming to digest. Low fibre forage will be readily and rapidly digested, leaving the gut empty and ready to digest further grazing, thereby rendering maximum nutritional rewards for the minimum digestive cost. Thus herbivores are likely to graze selectively so as to optimise digestive foraging (Belovsky and Schmitz, 1991).

A high incidence of sclerophylly is characteristic of fynbos vegetation. Sclerophylly is usually proposed to be an adaptation to facilitate nutrient use efficiency in the fynbos environment (Stock *et al.*, 1992). Sclerophylly increases leaf longevity and is

associated with evergreenness, thus leaf nitrogen is retained and functions in photosynthesis for long periods (although the rate of photosynthesis of sclerophyllous leaves is low; Reich *et al.*, 1992; Stock *et al.*, 1992). Sclerophylly has been suggested to be an adaptation to drought, but the correlation with aridity is not consistent (Stock *et al.*, 1992). However the band of lignified tissue in restioids is understood to prevent collapse of the culm following a loss of turgidity in the parenchyma cells, during seasonally dry periods (Linder, 1991). The deterrent value of sclerophylly against common dominant fynbos herbivores suggests that herbivory may be an important selective force in the evolution of high levels of sclerophylly fynbos vegetation.

Trade-off between tannin and sclerophylly

The positive correlation of herbivory with protein precipitating tannins seems initially paradoxical. Sclerophylly however, was inversely correlated with protein precipitating tannin quantity (Fig.11). Less sclerophyllous restioids were preferentially grazed, indicating that sclerophylly acts as a quantitative defence against herbivory. Thus there appears to be a trade-off between one type of defence (tannins) and another (sclerophylly). There is some evidence for a trade-off between the incidence of spinescence and tannin concentration in fynbos plants (Carrick, 1993). Both tannin and sclerophylly are carbon based defences, thus plants may allocate carbon to either defence strategy. Restioids lacking the genetic capacity to produce highly sclerophyllous culms, invest more heavily in tannins.

The carbon-nutrient-cycle hypothesis (Baas, 1989), holds that carbon will be produced in excess by plants in the nutrient deficient fynbos environment. This however is equivocal. Coley et al. (1985) maintain that the high defensive allocation of plants in nutrient deficient environments is an evolved trait. A trade-off in allocation to carbon-based defences indicate that carbon is not super-abundant but that carbon based compounds represent a cost to the plant, which is not supportive of the carbon/nutrient cycle hypothesis.

Tannin as a defence

Some tannins (probably of low molecular weight) and degraded products of tannins are known to diffuse across gut walls and act as toxins to herbivores. Tannins may also be toxic to gut microbes, impairing digestion by this means (Freeland and Janzen, 1974; Zucker, 1983). However the main defensive function of tannins against herbivores is to bind to proteins. Hence digestibility and nitrogen (protein) absorbence is reduced in two ways. Firstly, tannin binds to plant protein, released from the tissue by digestion, and precipitate it. Secondly, tannins bind to digestive enzymes within the gut, thus rendering them non-functional (Hagerman and Butler, 1991; Bernays et al., 1989; Swain, 1977).

Given the defensive functions of tannins, why then are these restioid tannins not effective at reducing *Otomys* grazing? Rodents are known to produce salivary, tannin binding proteins. Not all mammals have this ability. Rats and mice produce proline-

Concentrating tannin in the epidermis_x may be most effective since it represents the first line of defence against any form of attack. Herbivores and pathogens must first breach the epidermis in order to gain access to the underlying tissues (Swain, 1977; Zucker, 1983). Due to the high concentration of enzymes and proteins associated with photosynthesis, the chlorenchyma is likely to be the most nutritious tissue. Positioned in the epidermis however, tannins offer protection to this tissue from herbivores, such as phytophagous insects which may not devour the whole culm but merely feed on the outer nutritious layers, effectively scraping the butter off the bread. Such forage will obviously have a higher effective tannin concentration. Tannin in these restioids may therefore be a more effective defence against other forms of herbivory and possibly pathogen attack, than against mammalian herbivores.

Since tannin is restricted to the epidermis layer, it was thought that the higher tannin content in culm tops as opposed to bases (Figs.5,6) may be a product of the greater surface area/volume ratio (or transversely, the circumference/area ratio), associated with the smaller diameters of culm tops. However the culm top/culm bottom proportions were higher for tannin than for surface area/volume ratio values. This relationship is likely to be a factor of new growth. Culm tops represent the most recent growth of the plant, and new growth is generally more defended and has higher nitrogen contents than older growth (Bryant *et al.*, 1991; Hagerman and Butler, 1991).

Preference for plant parts

Although it was not possible to conduct quantitative feeding trials to determine preferences for the various restioid parts, and feeding behaviour of *Otomys* trapped at the study site, brief observations were conducted on two *Otomys* trapped in other areas. These observations confirmed a preferential avoidance of sclerophyllous restioids and culm parts (notably, *T. gracilis* and *W. glomerata*). *Otomys* appeared to be a highly selective (and wasteful) feeders, only consuming a small proportion of severed restioid culms. These rodents readily severed culms 5-10cm from ground level, thereafter passing the culm through their mouths until a suitable portion was found, upon which to feed. *Otomys* rarely consumed the bases of culms and were not observed to eat bracts, preferentially feeding on culm tops and other new growth (i.e. new shoots of branching culms) and also eating certain inflorescences (e.g. male *H. aristatus*). The higher sclerophylly (and possibly the lower nitrogen content) of the culm bottoms were likely to deter feeding.

Field observations also indicate that bracts seem to be rejected as forage. *Otomys* has the habit of gnawing grass and Restionaceae stems at various positions along their length, leaving smaller sections scattered on or near the runs (Curtis and Perrin, 1979; De Graaf, 1981; pers. obs.). A disproportionate number of these sections had bracts and often the stem appeared to be grazed up to the bract and then discarded (pers. obs.). The values for bracts alone (Figs.4-10) do not reflect the nature of the forage eaten by *Otomys*, since they eat the culm surrounded by a bract. Bracts have very low

nitrogen contents (Fig.4), and will thus lower the nutritional quality of the culm-bract forage. The bracts, with the exception of *H. aristatus*, virtually always constitute dead tissue. This is likely to account for the lower tannin content of these organs, since dead and older tissue generally has less tannin than new and actively photosynthesising tissue (Bryant et al., 1991). Tannin did not however act as a grazing deterrent in this study. The bracts of *H. aristatus* were often green and thus had higher succulence (Fig.8), tannin (Figs. 5,6), and water contents (Fig.7). Perhaps the greatest deterrent factor of bracts is the high fibre content. Although bracts were calculated to have a relatively low sclerophylly value, this measure is based on the mass/surface area ratio of the tissue. For the analyses however, bracts were separated from the culms and essentially hollow. Bracts are likely to contribute to the bulk of the forage but contribute virtually nothing to the nutritional quality.

Resource availability hypothesis

The results of this study did not support Coley et al. (1985) resource availability hypothesis. Neither herbivory or any defence character were found to correlate with the rate of growth of the restioid species.

The underlying driving force, namely resource availability, in this case the nutrient environment, was not tested implicitly. This study was specifically conducted in a single site in order to reduce environmental heterogeneity, thus nutrient availability is likely to be fairly similar. However the differential access of the various plants to

nutrients, based on the individual efficiency of root uptake, and possibly root mutualisms, is likely to be mirrored in growth rates. More pertinently, it is evolved growth rates (possibly determined by historic nutrient environment) that govern an individual plant's allocation to defence (Coley *et al.*, 1985; Coley, 1988). Deriving correlative trends from this data was hampered by the lack of spread of the growth rates, three species being clustered around 16% (Table 2). Coley *et al.* (1985) hypothesis may have application in fynbos at a larger (biome) scale, since the high incidence of sclerophylly may represent a defensive adaptation in this nutrient poor ecosystem.

Testing Grubb's (1992) hypothesis

The nitrogen content of culms were not related to the incidence of herbivory or any measure of defence. The data do therefore not support either contention, that in fynbos vegetation herbivores feed selectively on plants with higher nitrogen or that these plants will be more defended (Grubb, 1992). Culm nitrogen concentrations of the five species were however, extremely similar, ranging from 5.37mg.g⁻¹ to 6.64mg.g⁻¹, four having values above 6mg.g⁻¹. All nitrogen values (Fig.4) however, fell in the range (below 10mg.g⁻¹) inferred by Grubb (1992) to be too low to support the growth of herbivores. These nitrogen values were similar, but towards the lower end of nitrogen values reported for a number of other fynbos dicotyledons (Carrick, 1993). Tannin contents were however markedly lower than those of dicotyledons from similar vegetation (Glyphis and Puttick, 1988; Carrick, 1993). The fact that *Otomys*, possibly

the dominant grazer in mountain fynbos feeds avidly on such nitrogen-poor forage undermines the validity of Grubb's (1992) hypothesis. Furthermore, other studies have not shown support for this hypothesis (Carrick, 1993).

Other possible determinants of herbivory

Succulence and water content proved ~~not~~ to be poor correlates of herbivory in this study. Water content may be more important summer. *Otomys* is often found in vlei habitats (Skinner and Smithers, 1990), as was the case in this study, however all sampling was conducted over the wet winter months when the restioid species appear to contain near maximal water content, most of the time. In summer the ground at this site will be drier and most restioids will have markedly lower water contents (Linder, 1991). Thus it is possible that the water content of forage may be an important criterion in dry seasons, and *Otomys* may show an altered seasonal species preference.

It is possible that other characters fulfil defensive roles in restioids. Two other potentially defensive characters were not quantified and their effectiveness at reducing herbivory remains uncertain. Silica deposits are found in many restioids (Linder, 1991), although these deposits are not as extensive as those in grasses (Peter Linder, pers. comm.). Silica may increase tooth wear, is alleged to reduce digestibility by restricting the accessibility of microorganisms to vegetative cells, and if absorbed into the blood, may cause kidney failure (Vicari and Bazely, 1993). Glycosides have been reported to occur in some genera of the Restionaceae (Harborne *et al.*, 1985). The

function of these compounds is not known but glycosides often act as a toxin, and therefore form a qualitative defence against herbivory (Swain, 1977).

Sexual dimorphism

Female culms, generally have both a greater diameter and specific mass (sclerophylly) than male culms. Sclerophylly and width measurements are however related, since culms of greater widths have a larger volume/surface area ratio and hence a higher measure of sclerophylly (g.dm^{-2}). Although this sexual dimorphism is only marginal, it is likely to have ecological implications and may be explained in at least three ways:

(1) Female culms may be more heavily defended in view of their greater and/or longer term investment in reproductive output.

(2) Higher culm sclerophylly and diameter may be an adaptation to increase structural rigidity, which may be necessary to support heavy inflorescences and seed loads. This seems unlikely, since males of four of the five species (*W. glomerata*, the exception) had larger fertile inflorescences than females. Furthermore male *H. aristatus* culms frequently bore more than one inflorescence while females bore only one (pers. obs.).

(3) Male-male competition or pollen competition may be intense and drive selection for more male inflorescence. This sexual selection leads to males compromising on structural rigidity and/or defence (which may have a cost in terms of natural selection) in order to enhance the likelihood of reproduction.

Conclusion

Defence allocation in the Restionaceae was not consistent with the predictions of the resource availability hypothesis at a local scale (Coley *et al.*, 1985; Coley, 1988). However there appeared to be a trade-off in carbon-based defences, between the incidence of sclerophylly (fibre) and protein precipitating tannins, thus implying that carbon is not produced in excess but incurs some cost to production. Nitrogen content was similar in the five restioids but was extremely low, below that suggested by Grubb (1992) to be capable of supporting the survival of a mammalian herbivore. Thus low nitrogen concentration does not necessarily offer a refuge from grazing in the fynbos. *Otomys* however appeared to be a highly selective feeder and is likely to take advantage of the relatively low incidence of sclerophylly, and possibly the higher nutritional quality, associated with new growth at the tops of the culms. Relatively low levels of tannin in these plants did appear to effectively deter grazing by *Otomys*, but may be effective against other organisms such as phytophagous insects and pathogens. A high incidence of sclerophylly, however, appears to act as defence against grazing by this herbivore.

It is difficult to separate defence characters from other functions in plants (Vicari and Bazely, 1993). Sclerophylly may represent an adaptation to deter herbivory. It is also likely to have evolved as a means of conserving water and retaining nutrients by fynbos plants. These functions are synergistic, and it is likely that the combined

selective forces of these functions have led to the high incidence of sclerophylly in the fynbos. Herbivory may however be a greater selective force in mountain fynbos than has previously been envisaged, and should not be ignored as a force contributing to the evolution of certain plant traits and characters, not least of which sclerophylly.

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Appendix:

Cellular localisation of tannin

In order to localise the distribution of tannins transversely within the Restionaceae tissues, culms were sectioned, stained and viewed under a light microscope. Top and bottom parts of fresh culms of each species were examined. The sectioning was done by hand under a dissecting microscope, since a microtome was unavailable at the time of sectioning. The Nitroso staining reaction (Stafford *et al.*, 1987) was followed. This method was chosen as it is a quick and effective method identifying intracellular condensed tannins specifically. The classical stain, safranin, is not specific for tannins (Chesselet *et al.*, 1992). The Nitroso reaction involves adding equal volumes (numbers of drops) of: 10% (w/v) sodium nitrite; 20% (w/v) urea; and 10% (v/v) acetic acid, in succession to plant sections. After 3-4 minutes, two volumes of 2N sodium hydroxide were added. Condensed tannins stain a red, or possibly brown, colour.

Radial diffusion assay for protein precipitating tannins

The method for the radial diffusion assay follows that of Hagerman (1987) except where otherwise stated.

Extraction

Restioid culms were separated into the three parts as described in materials and methods. A number of culms from one plant were used for each sample. These were frozen in liquid nitrogen and ground manually using a mortar and pestle. The use of fresh material minimizes chemical degradation and other artifacts associated with sample preservation (Hagerman, 1988). Furthermore, extraction from fresh plant material has recently been shown to yield higher levels of tannin than that from lyophilized or air dried material (Hagerman, 1988; Owen-Smith, 1993). Ground material was weighed exactly and extracted in 70% (v/v) aqueous acetone, using a solvent to tissue ratio of 1ml per 200mg plant tissue. 50% methanol was not used, as recommended by Hagerman (1987).

In trials conducted by Hagerman (1988), 70% Acetone was found, in all cases, to be a more effective extraction medium than either aqueous or acidic methanol. Acetone is a superior solvent because it inhibits interactions between tannin and proteins, thus preventing tannin from becoming bound to tissue protein during homogenisation. This solvent does not however interfere with the radial diffusion assay as it evaporates before the tannin-protein interaction takes place (Hagerman, 1988).

Preparation of plates

An aqueous buffer was prepared which consisted of 50mM acetic acid and 60uM ascorbic acid, adjusted to pH 5.0 with NaOH. Agarose (type I) was dissolved in this buffer in a 1% (w/v) ratio by heating the solution to boiling while stirring. The

solution was then stabilised at 45°C and 0.1% (w/v) bovine serum albumin (BSA) added. The temperature is critical as higher temperatures denature the protein and at lower temperatures the agarose will set. 9.5ml aliquots of this solution were then dispensed evenly into standard plastic petri dishes (8.5cm diameter) and allowed to cool on a flat surface. Once set, 4mm wells were punched into the gel with a leaf punch. Four wells were made in each petri dish.

Assay

The sample tissue and acetone (in screw-top culture tubes) was rotated for 1 hour on a labquake rotator to maximise the extraction. 24ul of the resultant extract were then applied directly to the wells in the agarose plates with a micro-pipette. Three successive 8ul aliquots were applied, allowing sufficient time between applications for the acetone to evaporate off, as the wells have a smaller volume of less than 24ul. The plates were then incubated at 30°C for 120 hours. The tannin in samples migrates through the gel, binding to the protein and leaving a ring of tannin-protein precipitate which is cloudy white in colour. The area, or more conveniently the square of the diameter of the ring, is thus proportional to the quantity of active protein-binding tannin in the sample. The diameter of each ring was measured using a calibrated eyepiece in a dissecting microscope. The average of two perpendicular diameter measurements was used in case of variation in the ring formation. After subtracting the diameter of the well, the square of the diameter was compared to similar values on the calibration curve, the corresponding tannic acid equivalent expressed per mg fresh plant material.

Tannic acid was used as a standard. Although this is a hydrolysable tannin, it was used as a standard so that results would be comparable with other studies. 5mg tannic acid per 1ml 70% acetone, and various dilutions there of, were used to construct a calibration curve (Fig.1.).

Vanillin assay for condensed tannins

Material previously frozen and ground was dried in an oven at 30°C (higher temperatures may cause structural changes to the tannin; van der Heyden, pers. comm.). The vanillin assay for condensed tannins was carried out following the revised method of Price et al. (1978).

The ground sample was weighed and placed into screw-top culture vials with absolute (100%) methanol in the ratio: 200mg ground material per 10ml methanol, and then rotated for 1 hour in a labquake rotator. Price et al. (1978) found 20 minutes rotation to be adequate, but due to possible differences in the rotation method, an hour was felt to be appropriate. The extract was then centrifuged for 10min in a desk top centrifuge to precipitate the solid plant material. Assays were performed on the supernatant which together with the reagents were maintained at a constant temperature of 30°C in a water bath. Two reagents are used: 0.5% (w/v) vanillin and 4% HCl (v/v) in absolute methanol (which must be made fresh daily); and 4% HCl in methanol. 5ml aliquots of each reagent are added separately to two 1ml aliquots of extract at 1 minute

intervals. After exactly 20min the absorbence of each is read at 500nm on a spectrophotometer. The 4% HCl in methanol reagent serves as a blank and the absorbence of this solution is subtracted from that of the other reagent, to control for the absorbence of plant pigments and any HCl reaction. The results were calibrated against a standard curve, constructed using known quantities of catechin (from 0 to 0.6mg catechin/ml methanol).

In most cases the extract had to be further diluted to bring the absorbence values down into the range of the standard curve.

Nitrogen

The total nitrogen content of each individual plant sample was determined colorimetrically, following a Kjeldahl digestion process (Smith, 1980). 0.1g of ground material was used for digestion. This, together with 1ml distilled water, 3ml sulphuric acid (H_2SO_4) with salicylic acid and Sodium thiosulphate in excess, was added to breakdown nitrate and nitrite to ammonium. Standards were placed randomly on the heating block and run concurrently in the batch, in place of plant material and water, 1-2ml solutions of $(NH_4)_2SO_4$ which contained nitrogen in the range of 0.25-3mg.ml⁻¹, were used. A Selenium catalyst in the form of a Selenium Kjeldahl tablet was also added and the solutions placed on a melting block at 150°C until all the water had evaporated. The temperature was then very gradually raised to 350°C and left until

digestion was completed (after four hours). The acid digest was thoroughly mixed with water while still hot (manually and using a vortex mixer) and the solution made up to 50ml. A 0.5ml aliquot of this solution was used for colour determination. To this was added: 25ml 0.12% EDTA (w/v); a 2ml reagent of equal quantities of 0.5% Sodium nitroprusside in water (w/v) and 10% phenol in 95% ethanol (w/v); and 3.5ml of 1.5% Sodium hypochlorite in an alkaline phosphate buffer (1:4 ratio). The solutions were thoroughly mixed, made up to 50ml with distilled water and absorbance read on a spectrophotometer after colour development (minimum of one hour). The absorbences were converted to Nitrogen equivalents using the equation for the standard curve (fig.2) and expressed in milligrams nitrogen per gram dry plant material (mgN.g^{-1}).

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