COMPARISON OF MICE, GUINEA-PIGS AND SHEEP AS TEST ANIMALS FOR BIOASSAY OF OESTROGENIC PASTURE LEGUMES

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SUMMARY

Eight clovers were tested for oestrogenicity by uterine weight response in sheep, mice and guinea-pigs.

The activity of orally administered oestrogenic clovers relative to stilboestrol was several times higher in the rodents than in sheep.

Interactions between plant genotype and animal species indicated that sheep responded mainly to formononetin, although it is likely that there were also responses to other substances. Mice and guinea-pigs appeared to respond mainly to genistein, and perhaps biochanin A.

Tests for oestrogens in pastures which use mice or guinea-pigs as test animals may therefore be misleading if extrapolated to sheep, and perhaps also to cattle.

OESTROGENS in pasture legumes have been assayed by various workers using mice, guinea-pigs or sheep (Alexander and Watson, 1951; Davies and Bennett, 1962; Flux *et al.*, 1964; Bennett *et al.*, 1967). No direct comparisons between the results obtained with different animals on the same range of compounds, or of legumes, have been reported, but inconsistencies in the results obtained by different workers suggest that phytoestrogens may differ in potency among animal species. Thus Millington *et al.* (1964) reported that the oestrogenic activity, assayed by teat length increase in wethers, of nine lines of subterranean clover was correlated with the formononetin content, but not with the content of genistein or biochanin A. Although uterine-weight responses have been obtained from subterranean clovers which are low in formononetin (Bennett *et al.*, 1967), high formononetin levels have been found to give consistently high responses. In contrast, a number of studies, including those of Bickoff *et al.*

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(1962), Wong and Flux (1962) and Flux *et al.* (1964), have shown formononetin to be inactive, or much less active than genistein, when fed to mice. When given parenterally as pure compound, genistein and biochanin A have oestrogenic activities in sheep, comparable to those observed in mice (Braden *et al.*, 1967). Formononetin was inactive parenterally in ewes but this could have been due to its low solubility (Batterham *et al.*, 1965).

Bennett *et al.* (1967) have found zero, slight or large oestrogenic responses in sheep fed clovers containing substantial amounts of biochanin A and genistein, but little formononetin. Further, Davies and Bennett (1962) found little or no activity in Mount Barker subterranean clover, although numerous analyses from many samples of this cultivar have estimated approximately 1% of the dry weight of leaves to be biochanin A. Additional results from Davies and Dudzinski (1965) suggests that genistein is not consistently inactive in the sheep since positive responses were obtained from feeding the cultivar Clare, which contains little formononetin or biochanin A, but much genistein.

In view of the possibility that sheep do not respond to the same compounds as do mice and guinea-pigs, and the inconsistencies among sheep bioassays, it seemed important to compare the responses of ruminants and non-ruminants, as tests animals, to a range of plants differing appreciably in chemical composition. Should an animal species × genotype interaction be found in such comparisons, it would cast doubt on the validity of extrapolation from laboratory rodents to ruminants. Were such an interaction to be negligible, the difficulties and cost of ruminant bioassay might be avoided.

MATERIALS AND METHODS

The various legumes were grown, with some irrigation in autumn, on an alluvial clay-loam at the Ginninderra Experiment Station, near Canberra. The site is of high natural fertility reinforced by applications of superphosphate.

Material for sheep was cut on September 24, 26 and 27, 1965 (Series 1) with a sickle mower and fed fresh in pens the same or the following day to sheep that had been fasted for the 24 hours prior to day 1 of the test. The botanical composition and moisture content of each batch were determined from samples of about 400 g and 150 g,

respectively. Approximately 450 g of pure clover dry matter was intended to be fed to each sheep for each of four days. As the moisture content was not known until two days post-feeding, and since some sheep did not consume all feed offered, this intention was not realized on all occasions. Nevertheless, since consumption of most clovers was almost complete on days after the first day of feeding, the relatively slight variations in intake within treatments have been ignored, because it has been found that uterine weight reaches the maximum response after red clover has been fed for two days only (Morley, Bennett and Axelsen, unpubl. data).

There were two replicates of the guinea-pig tests. For the first, a two-day supply of plant material was picked by hand on September 20 and 22, 1965, and forwarded in plastic bags by air to the Clunies Ross Laboratory where the first feed from each sample was given to the guinea-pigs the same day as it was picked. Material used the second day was kept at 4°C until used. Material for the second replicate was obtained from a hand-sorted sample of that fed to sheep.

Plant material for some mouse tests was plucked by hand on September 20, 1965 from swards. Three hundred grams of fresh material, mostly leaf, was picked into one litre of industrial ethanol, stored for 24 hr, mixed thoroughly with a blender, stored for a further 24 hr and Buchner fiiltered. The alcohol was evaporated, lipids extracted with petrol ether, and the aqueous phase evaporated to about 70 ml, then made up to 150 ml with absolute ethanol. A 10 ml subsample was taken for chemical analysis. At the Clunies Ross Laboratory the alcohol content was reduced by evaporation and 0.15 ml of the concentrated solution was fed by syringe to each mouse twice a day for tests lasting three or four days. There were four replicates of the mouse tests between October 26 and December 13, 1965. Material for the further tests was obtained from samples of that fed to sheep on September 27. Results of some earlier tests, in which some mice were adversely affected by excess alcohol, were discarded.

Additional tests (Series 2) with sheep and guinea-pigs were made of some lines from October 25 to 29, using the same techniques as earlier tests.

The chemical analyses of the various legumes given in Table 1 were done at the Institute of Agriculture, University of Western Australia, from material sent by air, in sealed containers, after being cooled to about 4°C.

TABLE 1: LEVELS OF ISOFLAVONES IN BIOASSAY MATERIAL

Legume	*Test**	*% Dry Wt* Formononetin	Genistein	Biochanin A	*ppm* Coumestrol
Ladino	Sheep & G.-pigs, Ser. 1	0.01	0.02	0.05	5
white clover	Mice (Extract)	0.01	0.00	0.00	4
Marrar	Sheep & G.-pigs, Ser. 1	0.02	0.67	0.26	0
sub. clover	Sheep & G.-pigs, Ser. 2	0.39	1.44	0.79	–
	Mice (Extract)	0.09	0.70	0.18	1
Yarloop	Sheep & G.-pigs, Ser. 1	1.04	1.35	0.38	0
sub. clover	Mice (Extract)	1.18	2.75	0.30	0
Red	Sheep & G.-pigs, Ser. 1	0.86	0.01	0.35	0
clover	Sheep & G.-pigs, Ser. 2	0.81	0.13	0.91	–
	Mice (Extract)	0.83	0.08	0.83	1
Tallarook	Sheep & G.-pigs, Ser. 1	0.54	0.70	0.88	0
sub. clover	Mice (Extract)	0.77	1.35	1.16	2
Bacchus	Sheep & G.-pigs, Ser. 1	0.02	0.06	0.65	0
Marsh	Sheep & G.-pigs, Ser. 2	0.17	0.45	0.89	–
sub. clover	Mice (Extract)	0.05	0.09	0.76	0
Dinninup sub.	Sheep & G.-pigs, Ser. 1	0.61	0.48	0.84	0
clover	Sheep & G.-pigs, Ser. 2	1.06	0.75	0.86	–
	Mice (Extract)	1.22	1.78	1.33	2
Dixie crimson	Sheep & G.-pigs, Ser. 1	0.01	0.00	0.00	2
clover	Mice (Extract)	0.20	0.30	0.00	1

*Series 1: Bioassays in September, 1965.
Series 2: Bioassays in October, 1965

Samples arrived in fresh condition at the laboratory within 30 hr of collection in the field. Thin layer chromatography of extracts from samples was used following the technique described by Beck (1964) and modified by Francis and Millington (1965a). The extracts for mouse tests were hydrolysed before analysis using the techniques of Murti and Stone (1961). Extracts for mouse tests were made from leaves rather than leaves plus petioles and stems. This, and the extraction and analysis procedures, could account for differences between the extracts and the material fed to sheep and guinea-pigs in Series 1. Differences between similar analyses of Series 1 and Series 2 probably reflect sampling errors. Such differences between samples have been found by Morley and Francis (1968).

TESTS

The uteri of ovariectomized aged (cast-for-age) ewes (Merinos with a few Corriedales) were weighed after 4 days of feeding on the test material. Eight ewes were used for each treatment in Series 1, and a further eight for Series 2. The technique was that described by Lamond and Southcott (1962). Additional groups were injected intramuscularly with 4 μg or 16 μg daily of stilboestrol in peanut oil. The source of stilboestrol used was the same for all tests with all animals.

Mouse and guinea-pig tests used 3- or 4-day uterine weight responses, with stilboestrol groups and controls. Immature mice, 9 to 11 g weight, were used. Guinea-pigs weighing 250 to 450 g were ovariectomized 2 to 3 weeks before the test. They were housed in single cages and fed 20 to 35 g fresh clover twice daily. They readily consumed this amount. Stilboestrol standards were 0.25, 0.5 and 1.0 μg per day for guinea-pigs, and 30% alcohol (control) and 0.045 to 0.12 μg total dose orally for mice.

In statistical analyses of results, the log uterine weights, adjusted for uterine score (Davies and Dudzinski, 1965) in sheep, or for body weight in guinea-pigs, were used as the dependent variable. The log transformation and covariance adjustment were used following statistical analyses of uterine weights and responses by Bennett and Dudzinski (1967). The log transformation was found to give homogeneous slopes of the response of sheep to stilboestrol (log dose). Since no clear relationship between uterine weight and body weight could be established for mice, no adjustments were made.

The stilboestrol treatments and controls were not strictly necessary to the main objective of this experiment. They were included so that it would be evident whether responses to plants lay below the region of maximum response to an oestrogen. As can be seen in Tables 2 and 3, the maximum test response differed little from, or was less than, the maximum calibration response. As was intended, within the range of stilboestrol doses given, the response of log uterine weight was approximately linear to log dose. Hence any large interactions discovered in the analysis of these results would not arise because some treatment responses lay outside a region of linear response, unless the response to stilboestrol differed from that to phytoestrogens.

TABLE 2: BIOASSAY RESULTS

	Sheep (k=*10*)			*Guinea-pigs* (k=*1,000*)		*Mice* (k=*10,000*)	
Legume	*Log k Uterine Wt (g)*	*Stilboestrol Equivalent (μg*)*	*95% Confidence Limits*	*Log k Uterine Wt (g)*	*Stilboestrol Equivalent (μg*)*	*Log k Uterine Wt (g)*	*Stilboestrol Equivalent (μg*)*
Yarloop	2.71	**32**	15–67	2.84	**273**	2.11	**188**
Marrar	2.55	**5**	1–12	2.82	**204**	2.06	**117**
Dinninup	2.70	**28**	13–58	2.75	**123**	2.05	**118**
Tallarook	2.62	**12**	5–27	2.78	**156**	2.02	**88**
Bacchus Marsh	2.53	**4**	1–9	2.70	**78**	1.94	**28**
Ladino white	2.44	**0**	0–3	2.53	**7**	1.90	**5**
Dixie crimson	2.38	**0**	0–1	2.53	**8**	1.92	**16**
Red clover	2.66	**19**	9–40	2.74	**98**	2.00	**59**

*Stilboestrol equivalent is the number of injected μg stilboestrol calculated to give the same response as 1 kg dry matter of test material. In this test mice were given stilboestrol *per os* so figures given should probably be multiplied by 3 to make them comparable with those for other species.

RESULTS

Potencies of Legumes Relative to Stilboestrol

Analyses of results from Series 1 are given in Table 2, which gives mean uterine weights and estimated stilboestrol equivalents (μg) per kilo of plant dry matter. The calibration data are given in Table 3.

Confidence intervals are not given for mouse and guinea-pig bioassays since insufficient information on slopes and scales was available. The requirements for valid bioassays could not be met completely in this experiment. Critical tests for linearity and parallelism of response of stilboestrol and plant oestrogens have not been made for the plant materials or animals species used. Hence comparisons of responses, between animals species, *relative* to stilboestrol, should be cautious.

Despite such limitations it is apparent that the level of activity, relative to intramuscular stilboestrol, is much higher for rodents than for ruminants. This difference suggests that much of the activity of oestrogenic compounds is lost in the rumen.

Plant and Animal Interactions

The results of Table 2 indicate a generally good correlation between responses of the three animal species. Nevertheless, Marrar appeared to give a small response in sheep relative to that in rodents, and results from Bacchus Marsh and red clover also seemed inconsistent. Therefore, additional tests were undertaken (Series 2) with these and Dinninup. Dinninup and red clover were

TABLE 3: STILBOESTROL CALIBRATION RESULTS
Daily Dose of Stilboestrol

	Sheep		*Guinea-pigs*			*Mice*		
	4 μg	*16* μg	*0*	*0.5* μg	*1.0* μg	*0*	*0.015* μg	*0.030* μg
Log *k* uterine weight (g)	2.63	2.71	2.46	2.69	2.78	1.89	2.10	2.20
Standard error	0.034	0.034	0.049	0.046	0.046	0.024	0.024	0.024

Note: Log *k* uterine weights adjusted by log uterine score for sheep, and log bodyweight for guinea-pigs. Standard errors for mean log coded uterine weights 0.036 to 0.38 for sheep, 0.046 for guinea-pigs, 0.024 or 0.025 for mice.

high in formononetin, but different otherwise (Table 1). Bacchus Marsh and Marrar were both low in formononetin; however, the former was high in biochanin A, but low in genistein, while the latter was low in biochanin A but high in genistein.

The results obtained from these four clovers, over all tests with each animal species, were analysed to test for interactions, within animal species, between test and plant line. As shown in Table 4, interactions were negligible. Consequently, the data within any replicate or bioassay could be standardized as a proportion of the mean of the replicate-animal group. This removed replicate and animal variation from the analysis of variance, but also removed, as was intended, complications in estimating interactions in a set of classes with disproportionate numbers.

TABLE 4

Tests for interactions (Log k uterine weight)^2 (k(sheep) = 10, k(guinea-pigs) = 1,000, k(mice) = 10,000)

Source	*Sheep*		*Guinea-pigs*		*Mice*	
	D.F.	*M.S.*	*D.F.*	*M.S.*	*D.F.*	*M.S.*
Tests	1	0.020	3	0.85	3	0.19
Varieties	3	0.100	3	0.02	3	0.05
Interaction	3	0.007	9	0.012	9	0.007
Residual	58	0.009	48	0.011	47	0.007

The means over both series of log (k uterine weight) for plant lines and animal species are shown in Table 5, and the analysis of variance of log (k uterine weight), expressed as a percentage of the replicate mean, is given in Table 6. The value of k has been chosen to facilitate comparisons among means.

In Table 6, the degrees of freedom and mean squares for plant species and their interactions, have been partitioned in order to test the comparisons in what appeared to us to be the most logical set.

This partitioning discloses one important interaction—the ruminant is not behaving in the same way as the rodent in the contrast between formononetin-rich and formononetin-poor plants. The only other significant interaction is relatively small. While it may reflect relative differences in the sensitivity of the rodent species to different isoflavones it is scarcely large enough to justify speculation.

TABLE 5

Mean (log k uterine weight g) for combined Series 1 and 2 (k (sheep) = 10, k (guinea-pigs) = 1,000, k (mice) = 10,000)

Animal	*Plant*			
	Marrar	*Bacchus Marsh*	*Red clover*	*Dinninup*
Sheep	2.59	2.54	2.69	2.69
Guinea-pig	2.59	2.52	2.52	2.59
Mouse	2.08	1.95	1.99	2.03

TABLE 6: ANALYSIS OF ANIMAL × PLANT INTERACTIONS
(Combined Series 1 and Series 2)

Source	*Contrast*	*D.F*	*Mean Squares*
Animals	—	2	2
Plants		3	177
	(M+BM-R-D)	1	161
	M-BM	1	345
	R-D	1	26
Interaction		6	66***
	(M+BM-R-D) × (RU-RO)	1	241***
	(M+BM-R-D) × (MO-GP)	1	12
	(M-BM) × (RU-RO)	1	39
	(M-BM) × (MO-GP)	1	76*
	(R-D) × (RU-RO)	1	28
	(R-D) × (MO-GP)	1	2
Residual		180	17

M=Marrar subterranean clover; BM=Bacchus Marsh; D=Dinninup; R=Hamua broad red clover; RU=Ruminant (sheep); RO=Rodent (mouse or guinea-pig); MO=Mouse; GP=Guinea-pig.
*$P<0.05$.
***$P<0.001$.

Coumestrol content was omitted from this discussion since the intake was unlikely to induce activity. According to Francis and Millington (1965b) forage containing less than 50 ppm of coumestrol is unlikely to cause an oestrogenic reaction. Further, results with pure chemicals (Braden *et al.*, 1967) have shown that coumestrol intraruminally has only about 7×10^{-5} times the activity of stilboestrol intramuscularly. At the level of feeding in these experiments (about 500 g dry matter) one microgram of stilboestrol would be equivalent to about 140 ppm coumestrol.

DISCUSSION

The results reported indicate quite clearly that, at least for the present, oestrogenicity for sheep (as indicated by uterine weight response) of pasture legumes cannot be reliably assayed by mice or guinea-pigs. This is a pity.

The interaction analyses provide circumstantial evidence that sheep are responding predominantly to formononetin, rodents to genistein.

But this is by no means the whole picture.

First, there is evidence, from assays with white clover in Canberra (Bennett *et al.*, 1967) of the presence of oestrogenic compounds other than those mentioned in this paper. Secondly, uterine weight responses in sheep were obtained from Marrar and Bacchus Marsh in this and in other tests, from these and other varieties which were also low in formononetin. Further, on some occasions, clovers with high concentrations of genistein and/or biochanin A have given low or zero activity, as already discussed. It is known, also, that pure genistein and biochanin A are oestrogenic when given intramuscularly or intraruminally, and these compounds, and formononetin, have been found (Lindner, 1967) in the plasma of sheep that have eaten clover containing them.

It seems unlikely that the responses of sheep and rodents to formononetin are similar relative to those to genistein or biochanin A. D. A. Shutt and A. W. H. Braden (unpubl. data) found much higher concentrations of formononetin in the plasma of guinea-pigs than of sheep, both of which had been fed red clover. They also found similar levels of equol, a breakdown product of formononetin. They showed equol to be oestrogenic in mice, although somewhat less potent than genistein. The concentration of biochanin A+genistein was considerably higher in the guinea-pig than in the sheep. Their results are consistent with the hypothesis that the activity of red clover in rodents is primarily due to biochanin A+genistein, and in sheep to formononetin+daidzein+equol, but they do not approach a critical test of that hypothesis.

Some varieties of subterranean clover may contain unidentified oestrogenic compounds, but until the role of the isoflavones, and their breakdown products in oestrogenic reactions has been elucidated, one cannot be convinced one way or another from the evidence at hand, nor can the plant genotype×animal species interactions be understood.

For the present, at least, it seems clear that uterine weight responses in mice, and guinea-pigs, cannot be accepted as critical evidence for or against oestrogenicity of the tested material for sheep, and probably not for cattle either.

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