110. A COMPARISON BETWEEN INTERLITTER AND INTRALITTER VARIATION IN RATS WITH RESPECT TO THE HEALING OF RACHITIC BONES BY VITAMIN D

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MANY workers consider that good experimentation demands the variation of only one condition at a time. This is impossible in most experiments and especially in biological assays. In these, large numbers of animals are needed and they vary greatly in many ways, even when the colony (e.g. of rats) has been fed and housed in as nearly as possible the same surroundings for many years. In order to reduce the variation to a minimum, workers have generally chosen their animals within exactly defined limits of age, weight, etc. in a vain endeavour to vary only one condition in their experiment. Fisher [1937] has forcefully pointed out the futility of this in his 'Design of Experiments' and has shown not only how information on more than one point may be obtained from a carefully planned experiment, but also how information on the interaction of influences may be obtained. We have lately applied his teaching to the arrangement of two of our experiments, one of which was primarily intended as a comparison between two preparations of irradiated ergosterol and the other as a vitamin D determination of three separate preparations. We so arranged the allocation of the doses to the rats that we were able eventually to compare interlitter with intralitter variation, a point on which most workers hold strong views without any direct evidence.

Experiment 1

Two preparations of irradiated ergosterol, A and B, thought to be of equal potency, were to be compared for vitamin D potency by means of the line test. It was decided to compare doses of 2.5, 5.0 and 10 mg. of the one with equal doses of the other, i.e. 6 doses in all. Eighteen litters (6×3) were used, each consisting of 6 rats. Sex was ignored as Coward & Key [1933] had shown that there was no correlation between sex and the healing brought about by vitamin D. The weight of the rats within each litter at the beginning of the experiment of course varied, and Coward & Key [1933] had shown that the amount of healing produced by a dose of vitamin D given after a severe rachitic condition had been developed was correlated with the initial weight. Therefore it was necessary to distribute the rats equally, not only according to the doses but also according to the initial weight. Hence in this experiment, doses in the order 10.0, 5.0, 2.5 mg. of one preparation, and 10.0, 5.0, 2.5 mg. of the other were assigned to the rats of the first litter strictly in ascending order of their initial weights. In the second litter the doses were assigned in the same order beginning with the rat of the second lowest weight, the rat of the lowest weight then receiving the

last dose of the series. In the third litter, the cycle was shifted one place again, and so on throughout the litters. In the end, the rats were distributed amongst the doses as nearly equally as possible according to their initial weights. They were equally distributed according to litters and doses. The line test was carried out in the usual way and the results are given in Table 1. All the rats in

Table 1. Comparison of two samples of irradiated ergosterol, A and B

2.5 mg. A		5 mg. A		10 mg. A		2.5 mg. B		5 mg. B		10 mg. B		Total	Aver.
Wt.	Healing	Wt.	Healing	Wt.	Healing	Wt.	Healing	Wt.	Healing	Wt.	Healing	healing	healing
52	0.75	51	1.5	50	0.375	58	0.375	54	1.25	53	1.5	A 5.750	a 0.96
53	0.375	51	0.75	49	1.5	49	0	57	0.75	55	0.25	B 3.625	b 0.60
54	0.5	54	2.0	52	2.0	51	0.75	48	2.25	55	1.75	C 9.25	c 1.54
59	1.5	58	1.0	56	2.25	54	0.75	53	1.25	53	2.0	D 8.75	d 1.46
52	1.0	63	1.5	60	3.0	59	1.0	54	1.5	52	2.25	E 10.25	e 1.71
55	2.25	49	2.75	62	2.5	57	1.5	56	3.5	56	3.25	F 15.75	f 2.63
55	2.5	55	3.75	52	4.25	57	2.0	57	2.0	56	2.5	G 17.00	g 2.83
52	1.75	52	1.75	52	1.25	51	1.5	54	2.25	53	2.25	H 10.75	h 1.79
59	1.0	57	2.5	57	3.25	56	1.25	56	2.75	60	3.25	I 14.00	i 2.33
54	2.75	51	1.0	50	2.25	49	0.75	48	1.5	48	3.75	J 12.00	j 2.00
49	0.5	61	2.25	58	3.5	57	1.25	55	2.0	50	1.5	K 11.00	k 1.83
48	1.0	48	1.75	56	3.25	54	1.0	54	2.5	53	2.5	L 12.00	l 2.00
58	3.0	57	2.5	53	2.25	64	3.75	64	4.5	63	3.75	M 19.75	m 3.29
57	1.0	53	2.0	52	2.75	50	1.75	61	2.0	61	3.0	N 12.50	n 2.08
56	1.0	56	2.5	54	1.75	49	1.5	49	1.5	58	2.25	O 10.50	o 1.75
59	1.0	57	1.75	56	3.5	55	2.0	54	1.75	52	2.5	P 12.50	p 2.08
48	2.0	59	4.5	59	2.75	57	3.0	56	3.5	52	3.75	Q 19.50	q 3.25
50	1.0	49	2.5	56	4.75	55	1.25	53	2.0	50	4.75	R 16.25	r 2.71
P	24.875	Q	38.25	R	47.125	S	25.375	T	38.75	U	46.75	M 221.125	
p	1.38	q	2.13	r	2.62	s	1.41	t	2.15	u	2.60		m 2.05
Pp = 34.32750						Aa = 5.5200							
Qq = 81.4725						Bb = 2.175							
Rr = 123.46750						Cc = 14.245							
Ss = 35.77311						Dd = 12.775							
Tt = 83.3125						Ee = 17.5275							
Uu = 121.55						Ff = 41.4225							
479.90311						Gg = 48.11							
Mm = 453.30625						Hh = 19.2425							
Difference = 26.59686						Ii = 32.62							
						Jj = 24							
						Kk = 20.13							
						Ll = 24							
						Mm = 64.9775							
						Nn = 26.0000							
						Oo = 18.375							
						Pp = 26							
						Qq = 63.375							
						Rr = 44.0375							
						504.5325							
						Mm = 453.30625							
						Difference = 51.22625							

any one line or row belonged to one litter. All the rats in any one column received the same dose. Then were calculated totals and averages for each row and each column, the product of each total and average, and the sums of the products for rows and columns respectively. Then the table was rearranged, keeping all the rats of any one litter in a row but putting the healing of the heaviest rat of each litter in the first column, that of the next heaviest rat in the second column and so on (Table 2). These columns were then totalled and averaged and the products (total $\times$ average) added together. Finally, the healing obtained in each rat was squared, all the squares were added together and the

Table 2. *Healing arranged according to initial weight of rats*

Heaviest rats ←→ Lightest rats											
Wt.	Healing	Wt.	Healing	Wt.	Healing	Wt.	Healing	Wt.	Healing	Wt.	Healing
58	0.375	54	1.25	53	1.5	52	0.75	51	1.5	50	0.375
57	0.75	55	0.25	53	0.375	51	0.75	49	1.5	49	0
55	1.75	54	0.5	54	2.0	52	2.0	51	0.75	48	2.25
59	1.5	58	1.0	56	2.25	54	0.75	53	1.25	53	2.0
63	1.5	60	3.0	59	1.0	54	1.5	52	2.25	52	1.0
62	2.5	57	1.5	56	3.5	56	3.25	55	2.25	49	2.75
57	2.0	57	2.0	56	2.5	55	2.5	55	3.75	52	4.25
54	2.25	53	2.25	52	1.75	52	1.75	52	1.25	51	1.5
60	3.25	59	1.0	57	2.5	57	3.25	56	1.25	56	2.75
54	2.75	51	1.0	50	2.25	49	0.75	48	1.5	48	3.75
61	2.25	58	3.5	57	1.25	55	2.0	50	1.5	49	0.5
56	3.25	54	1.0	54	2.5	53	2.5	48	1.0	48	1.75
64	3.75	64	4.5	63	3.75	58	3.0	57	2.5	53	2.25
61	2.0	61	3.0	57	1.0	53	2.0	52	2.75	50	1.75
58	2.25	56	1.0	56	2.5	54	1.75	49	1.5	49	1.5
59	1.0	57	1.75	56	3.5	55	2.0	54	1.75	52	2.5
59	4.5	59	2.75	57	3.0	56	3.5	52	3.75	48	2.0
56	4.75	55	1.25	53	2.0	50	4.75	50	1.0	49	2.5
<i>P</i>	42.375	<i>Q</i>	32.50	<i>R</i>	39.125	<i>S</i>	38.75	<i>T</i>	33.00	<i>U</i>	35.375
<i>p</i>	2.35	<i>q</i>	1.81	<i>r</i>	2.17	<i>s</i>	2.15	<i>t</i>	1.83	<i>u</i>	1.97

$$Pp = 99.58125$$

$$Qq = 58.825$$

$$Rr = 84.90125$$

$$Ss = 83.3125$$

$$Tt = 60.39$$

$$Uu = 69.68875$$

$$456.69875$$

$$Mm = 453.30625$$

$$\text{Difference} = 3.39250$$

total healing M was multiplied by the average healing m . The difference between the sum of the squares and Mm is the total variance of the experiment. It is analysed as follows:

$$\text{Sum of squares} = 572.42189.$$

$$Mm = 453.30625.$$

$$\text{Difference} = 119.11564.$$

		Degree of diff.	Mean squares	Variance ratio	Points of e^{2z}
Variance due to diff. between doses	=26.59686	5	5.31937	11.23	0.1 % = 4.65
Variance due to diff. between litters	=51.22625	17	3.01332	4.44	0.1 % = 2.95
Variance due to diff. between initial weights	= 3.39250	5	0.67850	1.43	5 % = 2.34
Variance due to randomization	=37.90003	80	0.47375		
	119.11564	107	1.1039		

One would expect the variance due to differences in doses when there are two series of three each in the ratio of 1 : 2 : 4 to be significant, as indeed this calculation has shown that it is, the variance ratio 11.23 being much greater even than the 0.1% of e^{2z} which for these numbers of animals is 4.65. The variance due to differences between litters is also highly significant, the variance ratio being 4.44, the 0.1% point being 2.95. The variance due to initial weights is of very doubtful significance compared with the others, as its variance ratio is only 1.43, the 5% point being 2.34. Hence there is ample evidence in this experiment that the intralitter variation in rats is much greater than the inter-litter variation, when that is judged by differences in weight at the beginning of the experiment.

Experiment 2

This was a determination of the potencies of a cod liver oil and two preparations A and B made from it, each of 50 % strength. The doses tested were 40 mg. cod liver oil, 80 mg. preparation A, 80 mg. preparation B, 3 and 9 units respectively of the International Standard. They were assigned to rats in descending order of initial weight as in the first experiment. Results were tabulated in a similar way and gave the following analysis:

Sum of squares = 143·17189. $Mm = 105·4878125$.
Difference = 37·68408.

		Degree of diff.	Mean squares	Variance ratio	Points of e^{2z}
Variance due to diff. between doses	= 4·152	4	1·038	2·44	5 % = 2·67
Variance due to diff. between litters	= 19·4151	9	2·157	5·06	0·1 % = 4·44
Variance due to diff. between initial weights	= 0·496	4	0·124	0·29	5 % = 2·67
Variance due to randomization	= 13·621	32	0·426		
	37·684	49	0·769		

The variance due to doses was small, for four of them proved to be of nearly equal potency, only one of them, 9 units, being very different from the rest. The variance due to differences between litters was, however, highly significant, the variance ratio being 5·06, the 0·1 % point being 4·44. The variance due to differences in initial weight was again very small, the variance ratio being 0·29, the 5 % point being 2·67.

SUMMARY

It has been shown, by carefully planning an experiment designed primarily for other purposes and analysing the variance according to the method proposed by Fisher, that the variation between litters of rats in response to vitamin D is much greater than the variation between rats of the same litter as judged by their weights when they were first given the rachitogenic diet. The results emphasize very strongly the importance of using 'litter-mate' comparisons in biological assays of vitamin D.

REFERENCES

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Fisher (1937). *Design of Experiments*, Chapter vi. Oliver and Boyd.