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Analysis of lines of mice selected for fat content. 2. Correlated responses in the activities of enzymes involved in lipogenesis

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Summary

Estimates of the activities (V_{max}) of six enzymes involved in *de novo* fat synthesis were made in replicated lines of mice differing in fat content. These lines had been selected high and low for 20 generations with three replicates each of Fat, Control and Lean lines and for a further eight generations high and low as an unreplicated line. The activities of ATP-citrate lyase (ACL), acetyl-CoA carboxylase (ACC), fatty acid synthetase (FAS), cytoplasmic malate dehydrogenase (MDH), malic enzyme (ME) and pyruvate kinase (PK) were determined *in vitro* in both liver and gonadal fatpad tissues taken at ages five and ten weeks. The activities of ACL, ACC, FAS and ME were significantly higher in the Fat than the Lean lines, and the differences were more pronounced at the earlier age and in the gonadal fatpad where activities in the Fat lines were higher by factors of 3·5, 2·4, 2·5 and 3·5 respectively. The activity of PK was unchanged in each tissue. MDH activity was significantly lower in adipose tissue in the Fat lines than the Lean lines at age ten weeks but not at age five weeks or in liver tissue. Results from replicates indicated that random genetic drift affected enzyme activities but nevertheless significant changes in activity were associated with the direction of selection. The changes in enzyme activity reported here are similar to those known to be associated with major mutations causing obesity in mice.

1. Introduction

This study utilizes replicated lines of mice divergently selected for fat content and bred as part of a larger programme of selection to create a resource for physiological studies (Sharp, Hill & Robertson, 1984). The divergent lines differ by factors of between two and three in estimated fat content with negligible changes in lean mass (Hastings & Hill, 1989), and are known to differ in their rates of lipogenesis (Asante, 1989). The fat synthesis pathway is a short, well characterized pathway with the advantage for analysis of having a well defined end point. The biochemistry and physiology of fat synthesis have been studied in depth, because of its commercial importance in animal production and its implications for human health. Asante, Hill & Bulfield (1989) measured the activities of enzymes producing NADPH, a cofactor essential in lipogenesis, and found significant differences between these lines. This study measured the activities of six enzymes involved in *de novo* lipogenesis: ATP-citrate lyase (ACL; EC 4.1.3.8), acetyl coenzymeA carboxylase (ACC; EC 6.4.1.2), fatty acid synthetase (FAS), cytoplasmic malate dehydrogenase (MDH; EC 1.1.1.37), malic enzyme (ME; EC 1.1.1.40) and pyruvate kinase (PK; EC 2.7.1.40). The lipogenic pathway contains enzymes exhibiting a range of functional features typical of metabolic enzymes such as hormonal regulation of activity (ACC), non-hormone regulation of activity (FAS), and isozyme variation between tissues (PK, in which activity is regulated by hormones in the liver but not in adipose tissue). It was hoped to identify some of the metabolic changes which contribute to the changed phenotype.

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2. Materials and Methods

(i) *Mouse stocks*

Three replicates of mice (named F1, F2 and F3) were divergently selected for 20 generations on the basis of fat content in 10-week-old males, estimated by the ratio of gonadal fatpad weight to body weight (Sharp *et al.*, 1984). Unselected control lines were maintained giving nine lines in all, three each of Fat, Lean and Control. This selection resulted in an average 2·5-fold difference between Fat and Lean lines in whole body fat content at 10 weeks (Fat = 16·5%, Control = 10.0%, Lean = 6·4%; Hastings & Hill, 1989). The F6 lines were a product of crossing these replicates at

generation 20 to produce the F6 Fat line (from the three Fat replicates) and the F6 Lean line (from the three Lean replicates). These crosses were intended to restore much of the genetic variability lost through random drift over the initial 20 generations, but were unreplicated and no control line was established.

After this crossing, the original F1, F2 and F3 lines were maintained without selection during the ten generations prior to this experiment and they retained a divergence in fatpad weight (Table 1). The corresponding body-fat contents estimated by phenotypic regression (Hastings & Hill, 1989) on water content of these mice were 13·7, 11·5 and 6·9% in the Fat, Control and Lean lines respectively. The F6 lines had undergone a further eight generations of selection (now using dry matter content in 14-week-old males as a criterion of fat content), resulting in a 3·9-fold divergence in the ratio of gonadal fatpad weight to body weight at 10 weeks of age (Table 1). This corresponded to mean fat contents, estimated from water content, of 17·2 and 7·3% in the Fat and Lean F6 lines respectively.

The mice were maintained *ad libitum* on Beta Diets' Rat and Mouse Diet No. 1. The lines are known to have diverged in food intake (Bishop & Hill, 1985) but were not artificially restricted to the same intake as we were primarily interested in understanding how the additional intake in the Fat lines is partitioned into fat in preference to lean tissue (as happens in an analogous set of lines derived from the same base population but selected on lean mass).

Male mice were used because they are less prone to variation caused by steroid hormones and selection on fat content was applied only on this sex. Two ages were used, 5 weeks which represents the time of high growth and 10 weeks which represents the onset of maturity and the age of selection in the original F1, 2 and 3 lines.

(ii) *Preparation of samples*

Mice were killed by cervical dislocation between 10 and 11 a.m. to reduce any circadian variation in enzyme activity. Body weight was measured and the liver and gonadal fatpads immediately removed, weighed and placed in ice-cold homogenization buffer (0·25 M sucrose, 0·05 M Tris HCl pH 7·4, 1 mM EDTA and 1 mM dithiothreitol) in the ratio 1 g tissue to 3 ml buffer. This was homogenized and stored on ice. The samples were centrifuged at 17000 *g* for 40 min at 4 °C, the supernatant aliquoted into Eppendorf tubes and stored at −70 °C until use; preliminary studies of tissue extracts from both Fat and Lean lines had indicated that the enzyme activities were stable at this temperature. Liver and adipose tissue were taken as they represent major sources of lipogenesis in the normal mouse (22 and 7% respectively, Hollands & Cawthorn, 1981). Gonadal fat pad was the chosen source of adipose tissue because it is easily removed and was used to estimate total fat content during the first 20 generations of selection.

In the F6 lines 40 males from each direction were chosen, representing seven families from the Fat line and eight from the Lean. Half of each family was sacrificed at 5 weeks and the other half at 10 weeks of age. There was insufficient volume of fatpad tissue in several mice, particularly at 5 weeks, to enable all the subsequent assays to be performed, in which case gonadal fatpads were pooled, wherever possible with members of the same family. This resulted in twenty samples for 10 weeks Fat, twelve for 10 weeks Lean, fifteen for 5 weeks Fat and eight for 5 weeks Lean. The samples were assigned arbitrary numbers and all the subsequent storage and assays were performed blind.

In the F1, 2 and 3 lines, five samples were prepared from each replicate giving 45 in total. Each sample contained pooled material from between two and five mice, no more closely related than cousins, the same number being pooled for each of the five samples within each line. Where possible sibs were balanced so that half were killed at 5 weeks and the remainder at 10 weeks. Mice were sorted into cages of five individuals, one individual from each cage going to each pool. The samples were therefore balanced for family effects, cage effects and age of sacrifice.

(iii) *Biochemical methods*

Enzyme activities corresponding to each age (5 or 10 weeks) were determined on separate days using freshly made reagents. The samples from the F1, 2 and 3 lines were collected and assayed several months after the initial F6 assays, so some variation should be expected between assays. Each sample was measured three times, with the exception of FAS which was measured twice. Where necessary, dilutions of samples were made in standard homogenization buffer. ACL was assayed by the production of oxaloacetate from citrate (Linn & Srere, 1979), FAS by the production of NADP from NADPH (Carey & Dils, 1970). MDH by the production of NAD from NADH (Mosbach & Mattiasson, 1976), ME by the production of NADPH from NADP (Wise & Ball, 1964), PK by the production of pyruvate from phosphoenolpyruvate (Fitton, 1988) and ACC by the incorporation of $^{14}CO_3$ into malonyl CoA (Brownsey, Hughes & Denton, 1979). Determination of soluble protein in the homogenate was by a bicinchonic acid assay kit supplied by Pierce Chemical Company, Illinois.

(iv) *Statistical analysis*

In the F6 lines a simple *t* test using between-sample error was used. For the F1, 2 and 3 lines analysis of variance was represented by the model

$$Y_{ijk} = \mu + D_i + L_{ij} + e_{ijk},$$

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Table 1. *Body weights (BW), liver weights (LW), gonadal fatpad weights (GFPW) and ratios of gonadal fatpad weight to body weight*

	F6 lines				Mean F1, F2, F3 replicate lines			
	BW (g)	LW (g)	GFPW (g)	GFPW/BW (mg g^{-1})	BW (g)	LW (g)	GFPW (g)	GFPW/BW (mg g^{-1})
				5 weeks				
Fat	24·8	1·54	0·336	13·2	21·5	1·36	0·185	8·3
Control	—	—	—	—	21·9	1·45	0·152	6·7
Lean	24·7	1·47	0·154	6·1	21·3	1·33	0·096	4·4
				10 weeks				
Fat	36·0	1·99	1·10	30·5	33·7	1·86	0·833	24·3
Control	—	—	—	—	32·0	1·77	0·487	15·1
Lean	35·6	2·09	0·28	7·8	30·6	1·72	0·220	7·2

Table 2. *Ratios of mean enzyme activities in Fat to Lean lines (i.e. Fat/Lean), and the significance of the difference between the means (i.e. of Fat − Lean)*

Age (weeks)	Liver						Gonadal fat pad					
	ACL	ACC	FAS	MDH	ME	PK	ACL	ACC	FAS	MDH	ME	PK
						F6 lines						
5	1·52***	1·12	1·21**	0·99	1·29**	1·11	3·53***	2·37***	2·52***	1·09	3·51***	1·38*
10	1·05	0·97	1·04	1·02	1·13	1·03	1·53*	1·52*	1·46***	0·81***	1·57**	0·85
						Mean F1, F2, F3 replicate lines						
5	1·16	1·22	1·12	1·02	1·16	0·93	1·37***	1·16	1·26*	1·10	1·41***	1·02
10	1·21	1·19	1·15	1·04	1·38*	1·06	1·22	1·88**	1·40*	0·81***	1·33	0·75*

* $P < 0·05$; ** $P < 0·01$; *** $P < 0·001$.

where Y_{ijk} is the observation on the kth sample of the ijth line in the ith direction; μ is the overall mean; D_i is the effect of direction of selection (H, L, C); L_{ij} ($j = 1, 2, 3$) is the effect of individual lines, attributable to genetic sampling (drift); e_{ijk} is residual error. The term D_i, representing the direction of selection, can be partioned into two effects with one degree of freedom: (i) due to divergence between the Fat and Lean lines and (ii) due to the asymmetry of divergence compared to the control lines. The effects for direction of selection were tested against L_{ij} to take account of drift variation between lines.

3. Results

The Fat and Lean lines had similar body and liver weights at both ages (Table 1). The slightly higher body weight of the Fat lines can be explained as differential deposition of fat on a similar lean mass (Hastings & Hill, 1989). Large differences were apparent in the weight of gonadal fatpads. The ratio of gonadal fatpad to body weight was about twofold higher in Fat than Lean lines at 5 weeks of age, and almost fourfold higher at 10 weeks.

The results of the F6 line are treated separately from those of the F1, F2 and F3 replicates. This reflects differences in their selection history and the fact that collection, storage and assays of samples were performed separately for the two sets of lines. The divergences in activities between the Fat and Lean lines are the parameters of most interest and are given as the ratios Fat/Lean for each enzyme in each tissue at both ages in Table 2. Significance tests were made on the difference in activities between the Fat and Lean lines. Details of activities in each replicate for each enzyme, tissue, and age are given in Appendix Tables 1, 2 and 3, together with appropriate extracts from the analysis of variance in Appendix Table 4 and some comments on these specific activities.

The activities of ACL, ACC, FAS and ME were significantly higher in the Fat than the Lean lines, and the differences were more pronounced at the earlier age and in the gonadal fatpad, where activities in the F6 Fat lines were higher by factors of 3·5, 2·4, 2·5 and 3·5 respectively. The activity of PK was unchanged in each tissue. MDH activity was significantly lower in the Fat lines in 10 week adipose tissue but not at age 5 weeks nor in liver tissue. There are significant effects

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of random genetic drift ('lines' component of the analysis of variance in Appendix Table 4), but little asymmetry in response. The activities of each enzyme were measured for each individual mouse or pool of fatpad tissue so within-line correlations could be calculated between enzyme activities and estimated fat content. There appeared to be no consistent correlation between enzyme activity and fat content (data not shown).

4. Discussion

The largest and most consistent differences in activity occur in the three enzymes which constitute the pathway of *de novo* synthesis of fatty acids, i.e. ACL, ACC and FAS. Among the three enzymes indirectly associated with fat synthesis, only ME exhibits the same degree of divergence (Table 2), possibly due to its importance in generating NADPH, a substrate of FAS necessary for fatty acid synthesis. Levels of PK and liver MDH had not diverged, which may indicate a lesser influence on fat synthesis or possibly a greater degree of constraint imposed by their more diverse metabolic significance. A similar argument may explain why differences are more apparent in fatpad tissue. Liver has diverse metabolic functions and may be constrained by the changes it can tolerate whereas gonadal fatpad appears to have little metabolic significance beyond the synthesis, release and storage of fats so may not be under the same degree of constraint. The reason why MDH was lower in gonadal fatpad tissue from the Fat lines is unclear. One plausible explanation is as follows: activities were expressed per mg soluble protein extracted from the tissue so if the amount of ACC, ACL, FAS and ME protein had increased in the Fat lines (as suggested by the data), this will lower the specific activity of MDH.

Gonadal fatpad may not be representative of all adipose tissue since metabolic differences occur between different anatomical sites (Anderson, Kauffman & Kastenschmidt, 1972). It was used to estimate total fat in the selection index in the F1, 2 and 3 lines so may have been selected for increased lipogenesis with a decreased capacity for lipid release in the Fat lines and, conversely, decreased lipogenesis with increased mobilization in the Lean lines.

A criticism sometimes levelled at attempts to relate changed physiological traits with altered phenotype is that such experiments frequently use unreplicated strains of animals (Eisen, 1989). The presence of replicates is a prerequisite for investigating correlated changes to selection as they estimate the effects of random genetic drift (Hill, 1980). The results obtained here suggest that random genetic drift had significant effects on enzyme activities but when incorporated into the analysis of variance did not alter the conclusion that significant changes in activity had occurred as a consequence of the type of selection used to establish the lines.

Asante (1989) measured the relative rates of lipogenesis *in vivo* by the incorporation of tritiated water into lipids, in the same tissues at the same ages in the lines used in this study. Lipogenesis was elevated in the Fat lines relative to the Lean lines by factors of 1·14 and 2·15 at age 5 weeks, and factors of 1·22 and 1·45 at age 10 weeks for liver and fatpad respectively. These ratios are similar to those obtained in this study for the relative *in vitro* activities of lipogenic enzymes (Table 2). Asante *et al.* (1989) measured the activities (V_{max}) at ages 5 and 10 weeks in liver and gonadal fatpad tissue from the F1, F2 and F3 lines of four NADPH-producing enzymes: glucose 6-phosphate dehydrogenase (G6PDH), 6-phosphogluconate dehydrogenase (6PGDH), isocitrate dehydrogenase (IDH) and malic enzyme (ME). The largest differences were apparent in fatpad tissue and at age 5 weeks where activities were higher in the Fat lines by factors of 1·31, 1·11, 1·27 and 2·25 respectively. The degree of divergence in ME activity is similar to that obtained in this study and is the only one of the four where the divergence in activity *in vitro* is similar to the divergence in lipogenesis *in vivo*.

Activities of a range of enzymes are elevated rather than just one critical 'rate-limiting' enzyme as expected from recent analyses which stress that each constituent enzyme has an effect or potential effect on the overall rate of any biochemical pathway (Kacser & Burns, 1981). In particular the hormone-regulated enzymes ACC and liver PK do not appear to be elevated to a greater amount than the others. The results presented here indicate that all the enzymes directly concerned in lipogenesis show similar divergence in activity irrespective of their temporal role in regulating lipogenesis; for instance, ACC is modulated by several hormones *in vivo* to control the rate of lipogenesis but is no more divergent in activity than FAS which is not modulated. The view that attempts to improve livestock production by genetic manipulation should concentrate on classical 'rate-limiting' enzymes should therefore be regarded with some scepticism on the evidence reported here. Similarly, evolutionary change may be expected to proceed via changes in activity of several enzymes rather than by an accumulation of mutations in critical 'rate-limiting' enzymes.

The enzyme activity changes reported in this study are similar to those associated with obesity due to major mutations and environmentally induced obesity in mice. Chang *et al.* (1967) found elevated activities of hepatic ACC and FAS in obese *Ob/Ob* mice compared to their normal littermates. Bulfield (1972) found elevated levels of hepatic ACL, ME and PK in murine obesity caused by the *Ob* gene, the db^{ad} (previously known as the *Ad*) gene or an aurothioglucose-induced phenocopy, whereas MDH activity was unaltered. Kaplan & Fried (1973) reported elevated activity of MAL in adipose tissue of *Ob/Ob* mice. It appears that quantitative genetic selection

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resembles the effects of major gene mutations and phenocopy in their effect on enzyme activity underlying the increased deposition of fat.

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Appendix

Activities of individual lines for each enzyme, tissue and age

Some differences are apparent in activities between the results from F1, 2, 3 and F6 assays, i.e. at 5 weeks fatpad ACL is higher in F1, 2 and 3 than in F6 by a factor of between 3 and 8, liver MDH is higher in F6 than in F1, 2 and 3 by a factor of 4 and fatpad PK is higher in F6 than in F1, 2 and 3 by about 75% (Appendix Table 1 *vs.* Appendix Table 2). Similarly at 10 weeks, liver and fatpad PK are higher in the F6 than in F1, 2 and 3 by a factor of 6–7 (Appendix Table 1 *vs.* Appendix Table 3). Except in the case of the 10-week PK results these differences cannot be attributed to variation in the assays since fatpad and liver samples were run concurrently using the same assay reagents. In effect each tissue was a control for the other and any changes in assay sensitivity would be reflected in both tissues. The differences are therefore due to genetic factors such as drift (unlikely to be of this magnitude), or environmental fluctuations such as food batch differences which may alter energy intake. Changes in dietary intake or composition are known to cause altered activities in these enzymes (Grigor & Gain, 1983) and may have occurred here. For these reasons the relative differences between lines are more informative, and are given in the main body of the text in Table 2.

Appendix Table 1. *Enzyme activities per unit soluble protein (nmol min^{-1} mg^{-1}) in F6 lines, residual standard deviations (S.D.), standard error (S.E.) of difference estimated from residual S.D. and significance (Sig.) of the difference*

	Liver						Gonadal fat pad					
Enzyme...	ACL	ACC	FAS	MDH	ME	PK	ACL	ACC	FAS	MDH	ME	PK
						5 weeks						
Fat	68·9	344	18·8	13180	148	1980	62·8	2250	84·8	5910	612	1380
Lean	45·4	307	15·5	13270	114	1790	17·8	948	33·7	5400	174	998
S.D.	16·2	71	3·6	2748	34·0	439	17·8	525	15·8	1565	118	312
S.E.	5·1	22	1·1	869	10·7	139	7·8	230	6·9	685	51·6	137
Sig.	***	n.s.	**	n.s.	**	n.s.	***	***	***	n.s.	***	*
						10 weeks						
Fat	51·8	344	13·4	7530	154	1980	50·1	631	33·6	3250	154	615
Lean	49·5	355	12·9	7390	137	1930	32·8	416	23·0	4010	98	722
S.D.	12·9	83	2·0	567	49·2	489	17·4	226	7·6	440	43·1	184
S.E.	4·07	26·2	1·58	179	15·6	155	6·4	82·5	2·8	161	15·8	67·2
Sig.	n.s.	n.s.	n.s.	n.s	n.s.	n.s.	*	*	***	***	**	n.s.

n.s. $P > 0{\cdot}05$; * $P < 0{\cdot}05$; ** $P < 0{\cdot}01$; *** $P < 0{\cdot}001$.

Appendix Table 2. *Enzyme activities per unit soluble protein (nmol min^{-1} mg^{-1}) at 5 weeks for each F1, F2 and F3 replicate and their mean, residual standard deviation (S.D.) and standard error (S.E.) of differences between means estimated from the between-replicate line mean square*

	Liver						Gonadal fat pad					
Enzyme...	ACL	ACC	FAS	MDH	ME	PK	ACL	ACC	FAS	MDH	ME	PK
						Fat lines						
1	46·0	609	15·6	3750	124	1142	174	1660	49·4	4980	505	751
2	43·1	544	14·7	3530	103	1095	161	2360	45·0	4870	590	666
3	41·3	487	14·9	3920	129	1390	176	1560	55·8	4990	657	591
$\bar{X}$	43·5	547	15·0	3740	119	1209	170	1860	50·0	4950	584	670
						Control lines						
1	41·2	433	15·6	3840	124	1223	136	2280	43·6	4770	554	649
2	49·5	599	16·0	3470	112	1050	142	2500	50·3	4660	525	557
3	48·9	531	13·7	3450	111	1062	147	1470	47·2	5510	602	581
$\bar{X}$	46·5	521	15·1	3590	116	1112	142	2080	47·0	4980	560	596
						Lean lines						
1	35·6	457	12·2	3770	95	1381	128	1310	42·4	4460	381	619
2	31·3	387	13·1	3610	80	1176	117	1570	38·0	4540	418	671
3	45·3	497	15·0	3660	132	1353	128	1960	38·6	4610	445	672
$\bar{X}$	37·4	447	13·4	3680	102	1304	124	1614	39·7	4540	415	654
S.D.	5·4	68	1·90	287	15·1	153	20·0	460	5·74	605	71	141
S.E.	4·16	55·4	0·89	145	14·5	101·9	5·6	362	3·22	207	43·1	46·4

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Appendix Table 3. *Enzyme activities per unit soluble protein (nmol min^{-1} mg^{-1}) at 10 weeks for F1, F2 and F3 lines; otherwise as Appendix Table 2*

	Liver						Gonadal fat pad					
Enzyme...	ACL	ACC	FAS	MDH	ME	PK	ACL	ACC	FAS	MDH	ME	PK
						Fat lines						
1	42·1	392	8·90	5060	133·7	366	42·3	975	33·6	3390	178	94
2	49·8	480	12·17	4910	151·9	196	36·9	970	30·9	3330	206	92
3	32·3	289	8·48	5080	109·3	315	34·2	1044	31·4	3510	193	96
$\bar{X}$	41·4	387	9·85	5020	131·6	293	37·8	996	32·0	3410	192	94
						Control lines						
1	29·8	297	8·77	5210	74·1	285	27·6	679	24·0	3980	134	90
2	33·1	330	8·89	4870	89·0	203	24·5	709	23·0	4030	141	121
3	39·1	323	7·63	4820	86·4	177	49·1	1056	35·9	4270	240	119
$\bar{X}$	34·0	316	8·43	4970	83·2	221	33·7	814	27·6	4090	172	110
						Lean lines						
1	29·5	297	8·23	5150	89·3	277	35·5	606	25·1	4320	167	116
2	35·6	350	9·41	4640	95·9	263	24·6	439	20·4	4180	122	137
3	37·8	325	8·09	4750	100·2	289	32·6	584	23·1	4080	142	125
$\bar{X}$	34·4	324	8·57	4850	95·1	276	30·9	531	22·9	4190	144	126
S.D.	3·6	34	1·73	308	15·1	60	11·0	267	5·2	296	35	45
S.E.	5·1	47·5	1·03	166	11·1	49·3	7·1	108·4	3·61	101	30·6	9·7

Appendix Table 4. *Extract from analysis of variance. Ratios of mean squares of direction (with 1 D.F.) (H − L), asymmetry (1 D.F.) and individual lines (6 D.F.) to residual mean square (36 D.F). Direction and asymmetry tested against lines; lines tested against remainder*

	Liver						Gonadal fat pad					
Enzyme...	ACL	ACC	FAS	MDH	ME	PK	ACL	ACC	FAS	MDH	ME	PK
						5 weeks						
H − L	9·57	16·29	4·75	2·00	8·69	2·85	39·83***	2·24	24·38*	3·45	42·30***	0·09
Asym.	12·88	1·26	1·85	0·03	1·27	8·87	0·79	5·68	1·39	1·00	7·40	2·22
Lines	4·48**	4·98***	1·70	1·92	6·97***	3·31**	0·58	4·66**	2·36*	0·88	2·76*	0·82
						10 weeks						
H − L	29·15	26·02	4·07	2·32	43·78*	0·56	2·97	22·83**	23·07*	56·66***	14·30	3·76*
Asym.	11·42	13·45	2·04	0·14	39·97*	11·08	0·03	0·36	0·01	10·40*	0·09	0·00
Lines	14·87***	14·89***	3·38**	2·18	4·02**	5·09***	3·16*	1·24	3·61**	0·94	5·70***	0·35

* $P < 0·05$; ** $P < 0·01$; *** $P < 0·001$.

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