ENVIRONMENTAL EPIGENETICS

Environmental Epigenetics, 2017, 1–19

doi: 10.1093/eep/dvx019
Research Article

RESEARCH ARTICLE

A 'phenotypic hangover': the predictive adaptive response and multigenerational effects of altered nutrition on the transcriptome of *Drosophila melanogaster*

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Abstract

The Developmental Origins of Health and Disease hypothesis predicts that early-life environmental exposures can be detrimental to later-life health and that mismatch between the pre- and post-natal environment may contribute to the growing non-communicable disease epidemic. Within this is an increasingly recognized role for epigenetic mechanisms; for example, epigenetic modifications can be influenced by nutrition and can alter gene expression in mothers and offspring. Currently, there are few whole-genome transcriptional studies of response to nutritional alteration. Thus, we sought to explore how nutrition affects the expression of genes involved in epigenetic processes in *Drosophila melanogaster*. We manipulated *Drosophila* food macronutrient composition at the F0 generation, mismatched F1 offspring back to a standard diet and analysed the transcriptome of the F0–F3 generations by RNA sequencing. At F0, the altered (high-protein, low-carbohydrate) diet increased expression of genes classified as having roles in epigenetic processes, with co-ordinated down-regulation of genes involved in immunity, neurotransmission and neurodevelopment, oxidative stress and metabolism. Upon reversion to standard nutrition, mismatched F1 and F2 generations displayed multigenerational inheritance of altered gene expression. By the F3 generation, gene expression had reverted to F0 (matched) levels. These nutritionally induced gene expression changes demonstrate that dietary alterations can up-regulate epigenetic genes, which may influence the expression of genes with broad biological functions. Furthermore, the multigenerational inheritance of the gene expression changes in F1 and F2 mismatched generations suggests a predictive adaptive response to maternal nutrition, aiding the understanding of the interaction between maternal diet and offspring health, with direct implications for the current non-communicable disease epidemic.

Key words: environment; nutrition; *Drosophila*; epigenetics; transriptomics; mismatch

Received 5 June 2017; **revised** 9 October 2017; **accepted** 17 October 2017

Background

Exposure to aberrant or harmful environments during development and early life can be detrimental to later life health. From these observations is derived the Developmental Origins of Health and Disease (DOHaD) hypothesis [1–4], which seeks to explain why the period from conception to birth and the first few years of life is critical for determining life-long susceptibility to non-communicable diseases (NCDs). Many NCD phenotypes are thought to be caused by developmental perturbations that are a consequence of altered epigenetic marks [2, 5], induced by environmental exposure during critical periods of development [6]. Alteration to the epigenome regulates gene expression through DNA methylation, histone and chromatin modifications [7, 8] providing plasticity to the genome. Consequently, phenotypes under epigenetic regulation provide a pathway through which the genome can interact with the environment [9]. If the epigenetic modifications occur at a time during which they are able to affect the germ line, such modifications may also influence development of the offspring [6].

The interaction between the environment and the epigenome and the resulting phenotypic adaptations, coupled with the growing NCD epidemic, has led to the predictive adaptive response (PAR) hypothesis [10]. This hypothesis states that nature of the PAR is determined by the degree of mismatch between the foetal pre-natal and its ultimate post-natal environments. This mismatch results from the information that the foetus receives on environmental conditions while *in utero*, to which it will respond adaptively by programming its biology to expect that environment. If the actual post-natal environment matches the prenatal prediction, then the PARs are appropriate and disease risk is low; if they do not match then the PAR is inappropriate, and disease risk is increased. For instance, obesity has a distinct epigenetic profile. This pattern could be established in early life as a response to the maternal, foetal and/or early post-natal environment, and later-life nutritional mismatch could mean that the individual has been programmed inappropriately, leading to an increased risk of obesity and associated diseases later in life [11]. This implies that the epigenetic hallmarks of early-life exposures may be able to be maintained or 'stored' in such a way as to produce long-lasting effects [12].

Along with stress, drugs and environmental toxicants, one of the main factors that can cause epigenetic perturbation is nutrition; evidence from humans, supported by experiments in rodents, suggest that early-life nutrition can affect the long-term health not only of the individual but also of their offspring [13–15], potentially through epigenetic mechanisms [16–20]. Consistent with the DOHaD hypothesis, strong links exist between both maternal and early-life nutrition and cardiovascular disease [21], diabetes and obesity [22] along with asthma and allergy, autoimmune disease, cancer and mental health [23–26]. Inappropriate maternal nutrition in rodents has been linked to incorrect epigenetic 'priming' during foetal or post-natal life [27, 28]; in particular, experiments conducted in *Drosophila* show that diets high in carbohydrate content (sugar) have been shown to programme metabolic status and diabetes [29, 30].

Many nutrition-related phenotypes have been attributed to changes in epigenetic processes and a classic example of this is that of methyl supplementation, which influences coat colour in agouti mice [31]. Nutrition influences are separated into direct effects (on the mother/father themselves) and also indirect (on the offspring or maternal provisioning) effects. Direct effects can be exemplified by high-fat diets inducing obesity and metabolic syndrome, due to the methylation pattern of particular genes and promoters involved in body weight and adipocyte differentiation such as leptin [32] and peroxisome proliferator-activated receptor gamma (PPARγ) [33] and the differential DNA methylation detected in metabolic syndrome in both humans and rodents [34, 35]. Indirect effects on offspring metabolic phenotype can occur via maternal diet. For example, rodents exposed to high-fat diets *in utero* have altered epigenetic patterns and methylation status of particular genes, for example those expressed in and secreted by adipose tissue such as adiponectin and leptin genes [36]. Further, a high-fat diet during pregnancy and lactation can induce epigenetic modifications and differential expression of the μ-opioid receptor (involved in drug metabolism), and corresponding hypomethylation of the promoter regions of the gene, in mouse offspring [37]. Additionally, maternal protein restriction in rodents can cause hypomethylation of particular genes involved in metabolic processes in foetus and offspring such as those that regulate metabolism in the liver [38, 39], those that contribute to cholesterol and fatty acid metabolism [40] and those that regulate metabolic pathways that control lipid metabolism between the liver and adipose tissue [41] and can also affect methylation in the developing placenta [42]. Such epigenetic perturbation is not just limited to foetal and early life; the post-natal period is also susceptible to the epigenetic effects of nutrition. For example, hypermethylation of the promoter region of the anorexigenic neurohormone proopipmelanocortin occurs in overfed rats [43], and post-natal folic acid supplementation can lead to hypermethylation of peroxisome proliferator-activated receptor alpha (PPARα), a nuclear transcription factor gene [44]. This sensitivity of the epigenome to the effects of the environment (nutrition) also extends into adulthood, where epigenetic changes in response to nutritional changes have been observed in rats [45–47].

In addition to metabolic genes, altered nutrition appears to have broader genomic consequences. For instance, a study in dairy cattle showed that nutrition can also alter markers of inflammation and oxidative stress [48]; in rats, a protein-restricted diet in pregnancy leads to an increased susceptibility to oxidative stress in offspring [49], while in humans, a high carbohydrate diet increases the oxidative stress response [50]. A high-fat, high-carbohydrate meal can induce oxidative and inflammatory stress as reflected by increased reactive oxygen species (ROS) generation in both normal weight [51] and in obese people [52], suggesting that oxidative stress and inflammation are major mechanisms involved in metabolic disorders associated with obesity [53] and can also induce epigenetic changes [35]. Environmental stress can induce DNA and histone-modified changes in gene expression in organisms ranging from plants [54] to humans [55]. In terms of applicability to health, we know that low doses of ROS, from calorie-restricted or high-carbohydrate diets, promote health and lifespan in numerous species [56]. Thus, considering the above, along with the PAR hypothesis, it is likely that such biological responses to nutrition reflect the idea that induced epigenetic changes that underpin physiological change and aid in the adaptation of an individual, and potentially its offspring, to an adverse environment [57].

Thus, considering that gene-specific studies of altered nutrition have demonstrated broad and diverse genetic and epigenetic consequences, it is pertinent to apply this concept to the whole genome. Nutrition is commonly investigated as an environmental factor that is expected to influence the epigenetic landscape, and there are several examples in the literature of response to altered nutrition being inherited multigenerationally [27, 58–60]

and transgenerationally (F3 and beyond, [31]). As such, altered gene expression, via epigenetic marks in response to nutrition, coupled with the PAR hypothesis, could be the key to understanding the prevalence of obesity and metabolic syndrome. Here, we explore the PAR hypothesis and the ability of nutrition to affect gene expression at a whole-genome level by manipulating the diet of the fruitfly, *Drosophila melanogaster*, to investigate the extent to which gene expression is changed by differing levels of macronutrients. Previous research has shown that a high-sugar maternal diet can alter the body composition of larval *Drosophila* offspring for at least two generations [29], as well as demonstrating that nutrition is able to influence traits relative to metabolic syndrome, longevity and the immune response [30, 61, 62]. Further, evidence also exists of a multigenerational response to the maternal condition (immune challenge, maternal age, Nystrand and Dowling, 2014 [63]). Considering such traits and responses are often under epigenetic control, we predict that dietary manipulation will have broad consequences for the expression of genes involved in epigenetic processes.

Methods

Fly Husbandry

Drosophila melanogaster stocks used in this study were wild-type Canton-S flies from the Bloomington Drosophila Stock Center at Indiana University. *Drosophila* were cultured in a dedicated invertebrate laboratory using standard techniques. Briefly, flies were maintained in laboratory incubators at 25°C in a P Selecta HOTCOLD-C incubator. Larvae were reared on either a standard low-protein high-carbohydrate (LPHC, standard laboratory fly food) or a high-protein low-carbohydrate (HPLC) diet. These differential diets consisted of standard brewer's yeast (Health2000, New Zealand), sugar (New Zealand Sugar Company, Auckland, New Zealand) and cornmeal (Health 2000) in varying ratios (Table 1). Agar (A7002, Sigma-Aldrich, St Louis, MO, USA), propionic acid (Thermo Fisher, New Zealand, AJA693) and Nipagin (47889, Sigma-Aldrich, 10% w/v in 100% ethanol) were added in equal amounts. Gross energy (kJ/g) of both the LPHC and HPLC food types was determined by bomb calorimetry, and total protein content (%) was calculated using the total combustion method (Table 1) by the Institute of Food, Nutrition and Human Health at Massey University, Palmerston North, New Zealand.

Nutrition Experiments

Drosophila were manipulated under anaesthesia (CO_2) and initially raised on LPHC (low-protein, high-carbohydrate, standard fly) food. To enable mating, 50 female flies were segregated within 4 h of eclosion and incubated with 10–15 male flies, on LPHC food, for 24 h. Female flies were separated and incubated on either LPHC or HPLC diet. Female flies laid eggs in their specified food and the F1 offspring from the HPLC diet were either maintained on HPLC, or mismatched onto LPHC diet (Fig. 1). The further offspring then remained on those matched or mismatched diets, relative to the F0 generation. Thus, the biological mismatch relates to flies from an HPLC (non-standard) dietary background that are mismatched onto an LPHC background.

At each generation, RNA was extracted from female *Drosophila* at 5 days post-eclosion using a modified kit protocol (Supplementary File 1). Following the extraction process, RNA was stored at −80 °C until needed.

Table 1: Fly diet components and content information

Component	High protein	Low protein
Agar (g)	9	9
Cornmeal (g)	66.7	66.7
Sugar (g)	31.24	46.7
Yeast (g)	148.76	16.7
Propionic acid (ml)	6.6	6.6
Nipagen (ml)	5	5
Water (l)	1	1
Protein (%)	8	5.3
Gross energy (kJ/g)	0.9	2.1
Yeast:sugar ratio	1:0.2	1:2.8

Transcriptomic Experiments

Two F0 generation replicates from each of the LPHC and HPLC diets and two replicates from the F1, F2 and F3 generations with matched and mismatched diets were prepared, resulting in 16 total RNA samples submitted to the Otago Genomics and Bioinformatics Facility at the University of Otago (Dunedin, New Zealand) under contract to the New Zealand Genomics Limited for library construction and sequencing. The libraries were prepared using TruSeq stranded mRNA sample preparation kit according to the manufacturer's protocol (Illumina). All libraries were normalised, pooled and pair-end sequenced on 2 lanes of high-output

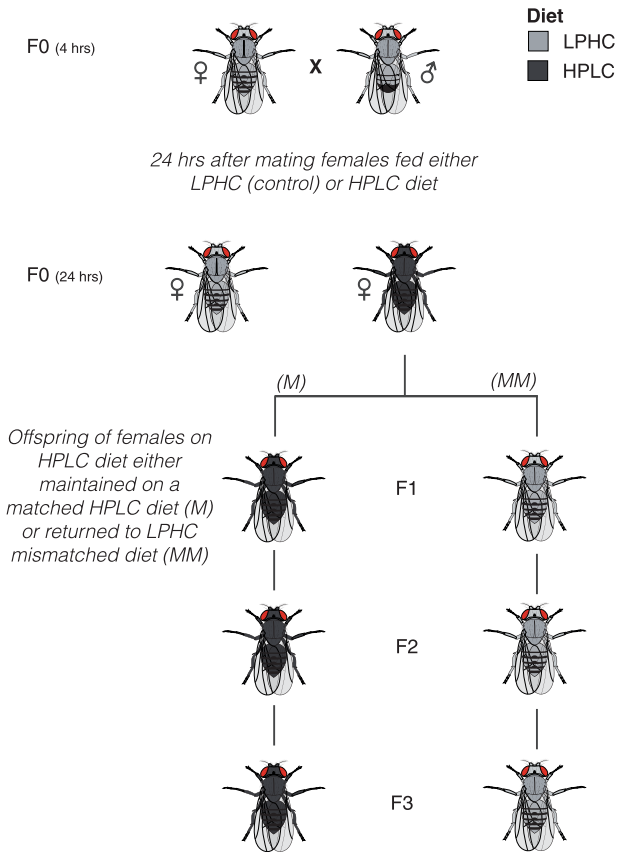

Figure 1: Fly diet experiments. LPHC, low-protein, high-carbohydrate (standard) diet; HPLC, high-protein low-carbohydrate diet; F1(M), F2(M) and F3(M), flies maintained on LPHC diet for three generations; F1(MM), F2(MM) and F3(MM), flies that were raised on HPLC in the F0 generation, and mismatched back to LPHC at eclosion in the F1 generation and were maintained for two more generations on the mismatched (LPHC) diet. Two replicates of each condition were used for transcriptomic experiments.

flowcell HiSeq 2500, V3 chemistry (Illumina), generating 100 bp reads. Libraries had an average insert size of ~208 bp.

Transcriptomic output was analysed in CLC Genomics Workbench Version 8.5.1. Reads were aligned to the *D. melanogaster* reference genome (BDGP6) as implemented in CLC Genomics Workbench, and differential gene expression (EDGE test) was calculated between samples using an absolute fold change value of >1.5, and an false discovery rate-corrected *P*-value of <0.001. These values were selected so that the fold change was of high enough magnitude that it could be validated in the lab by Nanostring, and the *P*-value was stringent to reduce false positives. Transcriptomic data were validated by Nanostring: samples were submitted to the Otago Genomics and Bioinformatics Facility at the University of Otago (Dunedin, New Zealand) under contract to the New Zealand Genomics Limited for nCounter Custom Gene Expression assays (Nanostring). Total RNA (100 ng) in a total volume of 5 μL was processed using the standard nCounter XT Total RNA protocol [64]. Raw data were exported and QC-checked using Nanostring's nSolver data analysis tool (www.nanostring.com). Per the Nanostring CodeSet design criteria, 25 candidate genes for validation were chosen, including two housekeeping genes incorporated (Mnf and Rpl32, Table 2). Raw data were normalized to the geometric mean of both the positive controls (included in the hybridisation steps) and the nominated housekeeping genes. Normalized Nanostring data were compared with transcriptomic data, and the Pearson's correlation coefficient was calculated in R [65].

Gene Ontology Analyses

Functional annotation clustering (FAC) was undertaken in the Database for Annotation, Visualization and Integration of Discovery (DAVID) v6.8 [66, 67] with the following categories: COG_ONTOLOGY, GOTERM_BP_DIRECT, GOTERM_CC_DIRECT, GOTERM_MF_DIRECT, KEGG_PATHWAY and INTERPRO. Up- and down-regulated genes from the F0 generation were submitted separately to DAVID for FAC and analysed against a background of genes that were expressed and detectable in this dataset, to identify gene oncology (GO) terms that were significantly enriched between the HPLC and LPHC F0 generation.

Table 2: Codeset design for Nanostring

Gene	Accession	Position	NSID
asf1	NM_079439.2	601–700	NM_079439.2: 600
Caf1-105	NM_136745.3	1231–1330	NM_136745.3: 1230
Def	NM_078948.2	8–107	NM_078948.2: 7
E(bx)	NM_167819.2	4681–4780	NM_167819.2: 4680
E(Pc)	NM_078974.2	2551–2650	NM_078974.2: 2550
E(spl)m4-BFM	NM_079786.1	241–340	NM_079786.1: 240
E(spl)m5-HLH	NM_079787.2	396–495	NM_079787.2: 395
Fbp1	NM_079341.1	3001–3100	NM_079341.1: 3000
Mnf	NM_168444.1	866–965	NM_168444.1: 865
Hml	NM_079336.2	7236–7335	NM_079336.2: 7235
Hmt4-20	NM_130497.2	3201–3300	NM_130497.2: 3200
Inos	NM_058057.4	1026–1125	NM_058057.4: 1025
Lip3	NM_057983.3	946–1045	NM_057983.3: 945
Lsd-1	NM_170092.2	836–935	NM_170092.2: 835
Lsp1beta	NM_057276.3	1351–1450	NM_057276.3: 1350
Ocho	NM_080514.1	416–515	NM_080514.1: 415
Pc	NM_079475.2	991–1090	NM_079475.2: 990
Pgm	NM_079936.2	841–940	NM_079936.2: 840
Rpl32	NM_170461.1	342–441	NM_170461.1: 341
Sap30	NM_132934.2	216–315	NM_132934.2: 215
Su(var)3-3	NM_140937.2	1781–1880	NM_140937.2: 1780
Su(var)3-7	NM_079618.2	3516–3615	NM_079618.2: 3515
Top2	NM_057412.3	3111–3210	NM_057412.3: 3110
Tps1	NM_134983.2	2056–2155	NM_134983.2: 2055
Ubx	NM_206497.1	1321–1420	NM_206497.1: 1320

Gene, gene symbol based on FlyBase nomenclature; NSID, Nanostring internal identifier; Position, region in the target mRNA being probed.

Statistical Analyses

Significant differences in mean gene expression between different dietary conditions and generations were calculated via analysis of variance (ANOVA) and Tukey's *post hoc* testing, as implemented in R [65].

Results

RNAseq Data

Summary statistics for transcriptomic work is shown in Supplementary File 2. Briefly, each sample yielded between 3164 Mbases and 4286 Mbases (average 4001 Mbases), with an average number of reads of 32 006 648 reads (range 25 310 758–34 288 584 reads). The mean quality score was an average of 36 Q (range 35.4–35.72 Q).

Differential Gene Expression

Of 17 490 genes annotated in the *Drosophila* genome and contained within the CLC reference database, 12 424 were expressed and detected across generations in these transcriptomic experiments. At F0, of these 12 424 genes, 2946 were differentially expressed between HPLC and LPHC [1074 (8.6%) down-regulated and 1872 (15.1%) up-regulated (Supplementary File 3)], with an absolute fold change of >1.5, and an FDR-corrected *P*-value of <0.001, as determined by EDGE test as implemented in CLC table.

Gene Ontology

DAVID uses a clustering approach to reduce redundancy; GO terms that are similar, are clustered together. Each term within the cluster is given a *P*-value, and the cluster itself is given an enrichment score (the geometric mean in –log scale of the individual GO term *P*-values). An enrichment score of 1.3 is equivalent to a non-log *P*-value of 0.05.

Functional annotation clustering of genes that are up-regulated in HPLC vs. LPHC indicated that the data set was highly enriched for genes that are involved in epigenetic processes such as chromatin binding [enrichment score (ES) 10.64], DNA replication (ES 4.64), chromatin regulation (ES 1.62), histone binding and phosphorylation (ES 1.60) (Table 3). Conversely, clustering of genes that are down-regulated in HPLC vs. LPHC indicated that the data set was highly enriched for genes that are involved in immunity (ES 11.41), fatty acid metabolism (ES 3.49), neurotransmission (ES 3.35), cellular metabolic processes (ES 2.49) and oxidative stress pathways (ES 2.19, Table 4). Table 5 lists the GO term classes and protein classes that are significantly up- or down-regulated in the F0 (HPLC vs. LPHC) generations, followed by those GO terms and protein classes that are up- or down-regulated in MM vs. M flies at each of the F1, F2 and F3 generations.

Table 3: Functional annotation clustering (FAC) as performed in DAVID

Annotation category	Term	Enrichment score	Gene	FlyBase gene ID	Function	P-value of ANOVA	Significance of ANOVA
Interpro	Zinc finger	10.64	Bre1	FBgn0086694	Ubiquitinates histone H2B on lysine 120 at most RNA Polymerase II transcribed genes	0.0304	*
			pygo	FBgn0043900	Binds His3 methylated tail to associate with arm as part of Wnt-induced transcription	0.0030	**
			e(y)3	FBgn0087008	PBAF complex, chromatin binding and gene silenecing	0.0596	.
			nej	FBgn0261617	Acetylates histone proteins and regulated gene expression	0.1420	NS
			E(bx)	FBgn0000541	Nucleosome remodelling, chromatin organization	0.0809	NS
			Pcl	FBgn0003044	Chromatin binding, co-localizes with the ESC/E(Z) complex	0.0439	*
Interpro/ GOTERM_MF_DIRECT	Zinc finger, DNA binding	4.64	E(var)3-9	FBgn0260243	Chromatin maintenance	0.0014	**
			Br140	FBgn0033155	Enhancer of polycomb-like	0.0027	**
			MTA1-like	FBgn0027951	Chromatin binding, chromosome condensation	0.0665	.
			spn-E	FBgn0003483	Chromatin binding, chromosome condensation	0.0153	*
			hang	FBgn0026575	Response to oxidative stress	0.0031	**
			phol	FBgn0035997	Polycomb group protein recruitment to polycomb response elements	0.0006	***
			Chd1	FBgn0250786	Remodelling and assembly of chromatin	0.0865	.
			Cp190	FBgn0000283	Chromatin binding	0.0165	*
			cg	FBgn0000289	Binds to polycomb response elements	0.0047	**
			su(Hw)	FBgn0003567	Negative regulation of chromatin silencing	0.0058	**
			Chd3	FBgn0023395	Chromatin assembly or disassembly, nucleosome remodelling	0.0027	**
GOTERM_BP/MF/ CC_DIRECT, INTERPRO	DNA replication, MCM complex	4.42	Orc1	FBgn0022772	origin recognition complex, DNA replication, chromatin binding	0.0013	**
			Orc4	FBgn0023181	Initiation of DNA replication	0.0136	*
			Orc2	FBgn0015270	DNA replication, chromatin binding	0.0004	***
			Mcm5	FBgn0017577	Chromatin binding, chromosome condensation	0.0008	***
			RecQ4	FBgn0040290	DNA stability, DNA rewinding	0.0047	**
			Mcm10	FBgn0032929	Heterochromatin organisation, chromatin silencing	0.0008	***
			polybromo	FBgn0039227	Chromatin binding	0.0104	*
GOTERM_BP/MF/ CC_DIRECT, INTERPRO	Microtublues, kinesin	4.05	cid	FBgn0040477	Histone H3 variant, epigenetic mark for centromere identity	0.0000	***

continued

Table 3: (continued)

Annotation category	Term	Enrichment score	Gene	FlyBase gene ID	Function	P-value of ANOVA	Significance of ANOVA
GOTERM_MF_DIRECT, INTERPRO	Helicase	3.10	XNP	FBgn0039338	Heterochromatin organization, chromatin silencing	0.0924	.
			me31B	FBgn0004419	Gene silencing by miRNA	0.0001	***
			Mi-2	FBgn0262519	Chromatin binding, nucleosome binding	0.0261	*
INTERPRO	Structural maintenance of chromosomes	2.61	SMC2	FBgn0027783	Chromatin binding, chromosome condensation	0.0020	**
			glu	FBgn0015391	Chromatin binding, chromosome condensation	0.0146	*
			SMC1	FBgn0040283	Chromatin binding	0.0263	*
			SMC3	FBgn0015615	Chromatin binding	0.0129	*
GOTERM_CC/BP_DIRECT	Rb-E2F complex, Myb complex	2.32	mor	FBgn0002783	Brahma associated proteins complex, PBAF complex	0.0100	*
INTERPRO, GOTERM_MF_DIRECT	Chromo domain	1.99	Chro	FBgn0044324	Histone binding, chromosome organization	0.0098	**
			Chd1	FBgn0250786	Remodelling and assembly of chromatin	0.0865	.
			Mi-2	FBgn0262519	Chromatin binding, nucleosome binding	0.0261	*
			msl-3	FBgn0002775	Methylated histone binding, chromatin binding	0.1760	NS
			Chd3	FBgn0023395	Chromatin assembly or disassembly, nucleosome remodelling	0.0027	**
GOTERM_MF/BP_DIRECT	Nucleosome mobilisation, nucleosome binding	1.76	dre4	FBgn0002183	Chromatin binding, nucleosome binding	0.0021	**
			E(bx)	FBgn0000541	Histone binding, chromatin organization, chromatin remodelling	0.0809	.
			Su(z)12	FBgn0020887	Polycomb repressive complex 2, histone methyltransferase activity'	0.0391	*
INTERPRO	WD40 repeat	1.67	Hira	FBgn0022786	Chromatin binding	0.0000	***
			Caf1-105	FBgn0033526	Chromatin assembly factor, histone binding	0.0001	***
			wds	FBgn0040066	Histone acetyltransferase activity, chromatin remodelling, Trx complex	0.0032	**
			ebi	FBgn0263933	Chromatin binding	0.0070	**
GOTERM_BP/CC_DIRECT	Histone phosphorylation	1.6	borr	FBgn0032105	Histone phosphorylation, chromatin binding	0.0006	***
			ball	FBgn0027889	Histone threonine kinase activity, histone phosphorylation	0.0009	***
			aurB	FBgn0024227	Chromatin organization	0.0004	***

Genes that were significantly up-regulated in these data in HPLC compared to LPHC were compared to a background list of genes that were expressed and detected in these data. Analyses of variance were carried out on expression between all samples in this study (F0-F3 matched and mismatched) with *P*-values and statistical significances listed.

Validation

Based on the genes that were up-regulated in this study, we selected a panel for 20 genes for transcriptomic data validation. All transcriptomic samples were validated by Nanostring, per-sample *r* value of 0.81–0.95 and whole data set correlation *r* of 0.86 (Supplementary File 4).

Multigenerational Gene Expression of Genes of Interest

Alteration of diet appears to lead to a characteristic suite of gene expression changes. To determine whether the suite of up-regulated genes observed in HPLC is maintained across mismatched generations when the HPLC diet is removed, we analysed the expression levels of particular groups of genes classed as having roles in epigenetic processes in F1, F2 and F3 matched (M) and mismatched (MM) flies. We observed a specific pattern of expression for every gene; the intermediate-level maintenance of the up-regulation of the genes in the F1 and F2 generation, followed by a reversion to F0 (matched) gene expression levels by the F3 generation. Fig. 2 displays a selection of indicative graphs which display this effect, with ANOVA significance data listed in Table 3 (described in Supplementary File 5) and significant pairwise comparisons as determined by Tukey's *post hoc* testing (Supplementary File 6) indicated by solid and dashed lines. There is no significant difference between the expression of epigenetic genes when comparing the F0 LPHC diet and the F3MM flies, despite an intermediate and significant difference between F1 and F2 flies mismatched onto LPHC diets. For genes that were significantly down-regulated in F0 HPLC flies, we observe the same pattern of gene expression in the opposite direction; genes that are down-regulated in response to diet remain at a low level in the F1 and F2 mismatched cohorts (F1MM and F2MM) but by F3, their gene expression has regained the same level as the LPHC (F0) generation (Fig. 3 and Supplementary Files 5 and 7). This effect is genomewide, and applies to every gene tested from the lists generated by DAVID.

Discussion

The primary goal of this study was to investigate the effect of nutrition on the expression of genes involved in epigenetic processes. We have demonstrated that an HPLC diet results in genomewide up-regulation of epigenetic genes in the F0 generation compared to that observed in a standard *Drosophila* LPHC diet; this effect was so strong that the overwhelming majority of genes that were up-regulated in response to diet had GO terms categorized as being involved in epigenetic processes, with very few other classes of genes categorized as significantly up-regulated. Classes of genes that were down-regulated in response to the HPLC diet vs. LPHC were broader in scope; these included genes that have GO terms with roles in the immune response, cell signalling, oxidative stress, carbohydrate and fatty acid metabolism and neurotransmission. Thus, this study shows that altering an LPHC diet to an HPLC results in genomewide up-regulation of genes classified as having roles in epigenetic processes, with a co-ordinated down-regulation of genes classified as having broad physiological functions. The co-ordinated nature of the gene expression data we observed imply, firstly, that genes with GO terms categorized as having roles in the processes of neurotransmission, oxidative stress, metabolism and immunity appear to be under epigenetic control, and, secondly, that this epigenetic control, and altered biological response, is influenced by nutrition.

In response to the genomic and epigenomic changes observed in the F0 generation, we further questioned whether dietary alteration resulted in developmental programming of the biological response to diet; specifically, whether the changes induced by the HPLC diet in the F0 generation persisted beyond F0, upon removal of the HPLC diet. The genomic changes induced in the F0 generation persisted at intermediate levels in mismatched F1 and F2 generations in the absence of the HPLC diet. By the F3 generation, gene expression in the mismatched flies had reverted back to the level observed in the F0 matched generation. We hypothesize, firstly, that this multigenerational inheritance of gene expression, followed by a reversion to matched F0 levels by the time the F3 generation is reached, indicates moderate epigenetic programming in the form of a predictive adaptive response (PAR); the gene expression changes induced by the dietary environment experienced by the F0 female flies may be maintained by her offspring. This intermediate maintenance may be considered either as an adaptive response to an environment that the F1-F3MM offspring are ultimately not experiencing or maintenance could be considered a form of bet-hedging, to mean that the offspring are primed to equally respond if their environment changes. Given that these data display a reversion to the unchallenged nutritional state after three generations, we further hypothesize that the 'correction' of the induced genomic changes by F3 implies that dietary reversion to match the F0 generation may be able to correct an altered genomic landscape, effectively reversing an altered nutrition-induced phenotype.

The environment is able to interact with genes through epigenetic mechanisms [9] particularly during development [2–4]. Crucially, this could also lead to the alteration of the epigenome of the germ cells [6]. Any permanent alteration to the germ cell epigenome [68] may then be transmitted through the germ line, with adverse phenotypic consequences for offspring [1, 69]. For example: adult-onset diseases can be induced through embryonic exposure to environmental toxins, primarily endocrine disruptors [5, 70–72]; toxic stress can modify *Drosophila* development by the suppression of Polycomb group genes, with epigenetic inheritance of developmental alterations by unchallenged offspring [73] and; mutations in chaperone proteins such as Hsp90 can induce a heritably altered chromatin state in *Drosophila*, suggesting a transgenerational epigenetic phenotype [74]. Thus, if epigenetic modifications do become permanent, these modifications can be inherited by future generations and affect disease susceptibility [58, 75].

A large number of studies report transgenerational inheritance in a range of eukaryotes (reviewed in [76]). Many of these studies, particularly in mammals, report inheritance of the acquired trait over two or three generations. Concordant with the work by Jirtle and Skinner [58], we agree that these effects should not be defined as truly transgenerational, because, mechanistically, exposure of an F0 gestating female to an environmental stimulus (nutrition, toxicants or stress) also exposes the F1 embryo (Fig. 1, [77]). Furthermore, for species that develop *in utero*, parental exposure also exposes the germ cells that will form the F2 generation. Thus, traits present in the F2 generation should be considered as multigenerational, rather than transgenerational, as they could have been induced by direct environmental exposure through the foetus and the germ line. This concept is equally applicable to *Drosophila* because, while they do not gestate, they do harbour ovarioles in their ovaries which contain developing follicles or egg chambers [78], thus their F1 eggs are exposed to the maternal environment. Further, F1 larvae, which in these experiments were raised on an HPLC diet before being mismatched back to LPHC, contain ovaries and germ cells for the F2 generation. Thus, to reflect this

Table 4: Functional annotation clustering (FAC) as performed in DAVID

Annotation category	Term	Enrichment score	Gene	FlyBase gene ID	Function	P-value of ANOVA	Significance of ANOVA
INTERPRO	Immunoglobulin-like domain/fold	11.42	mesh	FBgn0051004	Ig fold	0.000611	***
			Ppn	FBgn0003137	Ig domain, extracellular matrix structural constituent	0.0101	*
INTERPRO/ GO_TERM_BP_DIRECT/ GOTERM_MF_DIRECT	Proteolysis, peptidase	6.16	Jon65Ai	FBgn0035667	Serine-type endopeptidase, proteolysis	0.00293	**
			MP1	FBgn0027930	Serine-type endopeptidase, proteolysis	0.00187	**
			thetaTry	FBgn0011555	Digestive enzyme with serine-type peptidase activity	1.68E-08	***
			zetaTry	FBgn0011556	Digestive enzyme with serine-type peptidase activity	0.000488	***
			Decay	FBgn0028381	Cysteine-type endopeptidase activity, caspase	0.00188	**
			Damm	FBgn0033659	Cysteine-type endopeptidase activity, caspase	6.79E-06	***
			alphaTry	FBgn0003863	Serine-type endopeptidase, proteolysis	5.69E-05	***
			Ser7	FBgn0019929	Serine-type endopeptidase, proteolysis	1.51E-12	***
			Ser6	FBgn0011834	Serine-type endopeptidase, proteolysis	5.17E-03	**
			Swim	FBgn0034709	Polysaccharide binding, cysteine-type peptidase activity, proteolysis	1.35E-02	*
			Jon74E	FBgn0023197	Serine-type endopeptidase, proteolysis	1.58E-03	**
			psh	FBgn0030926	Peptidase and serine-type endopeptidase activity, defense response	4.41E-08	***
			Jon65Aiv	FBgn0250815	Serine-type endopeptidase, proteolysis	1.74E-04	***
			Jon65Aiii	FBgn0035665	Serine-type endopeptidase, proteolysis	6.77E-05	***
			Ance-4	FBgn0033366	Peptidase	6.97E-04	***
			Ance	FBgn0012037	peptidyl-dipeptidase activity	6.97E-04	***
			Jon25Bii	FBgn0031654	Serine-type endopeptidase, proteolysis	8.62E-04	***
GOERM_BP_DIRECT/ INTERPRO/ GOTERM_MF_DIRECT	G-protein-coupled receptor signalling, rhodopsin-like	6.02	Rh3	FBgn0003249	G-protein coupled photoreceptor activity	3.73E-03	**
			AkhR	FBgn0025595	Ccarbohydrate, lipid and tryglyceride homeostasis	1.88E-09	***
			Rh2	FBgn0003248	G-protein coupled photoreceptor activity	4.65E-02	*
			Galphas	FBgn0001123	Signal transduction, chemical synaptic transmission	1.07E-11	***
			ninaE	FBgn0002940	G-protein coupled photoreceptor activity, rhodopsin-like	2.80E-05	***
			Ggamma1	FBgn0004921	G-protein, gamma subunit, signal transducer activity	4.16E-05	***
			Gbeta76C	FBgn0004623	G-protein coupled photoreceptor activity	2.71E-02	*
			Rh4	FBgn0003250	G-protein coupled photoreceptor activity	1.83E-03	**
GOTERM_MF_DIRECT/ GOTERM_BP_DIRECT	Fatty acid elongation	3.49	eloF	FBgn0037762	Fatty acid elongase	4.60E-05	***
GOTERM_MF_DIRECT	Neuropeptide hormone activity/binding	3.35	Nplp2	FBgn0040813	Neuropeptide signaling, humoral immune response	1.39E-02	*
			Nplp3	FBgn0042201	Neuropeptide hormone activity, neuropeptide signaling	3.45E-01	NS
			Neb-cGP	FBgn0083167	Hormone activity, regulation of growth	3.72E-02	*
GOTERM_MF/CC/ BP_DIRECT	Calcium-dependent phospholipid binding	2.72	AnxB9	FBgn0000083	calcium ion binding, calcium-dependent phospholipid binding	7.69E-03	**
		2.5	Cals	FBgn0039928	Calcium ion binding, chemical synaptic transmission	2.29E-02	*

continued

Table 4: (continued)

Annotation category	Term	Enrichment score	Gene	FlyBase gene ID	Function	P-value of ANOVA	Significance of ANOVA
GPTERM_MP_DIRECT, INTERPRO, COG_ONTOLOGY	Calcium ion binding, signal transduction, EF-hand domain		Gel	FBgn0010225	Actin binding, calcium ion binding	1.52E-03	**
			Scp2	FBgn0020907	Calcium ion binding	3.43E-02	*
			Scp1	FBgn0020908	Calcium ion binding	8.74E-02	.
			Mlc2	FBgn0002773	Calcium ion binding	3.33E-04	***
			Zasp52	FBgn0265991	Muscle development	3.91E-08	***
			LpR1	FBgn0066101	Uptake of neutral lipids from circulation, calcium ion binding	4.93E-01	NS
			Mlc1	FBgn0002772	Calcium ion binding, myosin	3.91E-04	***
			up	FBgn0004169	Calcium ion binding, calcium ion homeostatis, muscle morphogenesis	3.26E-04	***
			TpnC73F	FBgn0010424	Calcium ion binding	3.01E-10	***
			Gpo-1	FBgn0022160	Glycerol-3-phosphate dehydrogenase activity, calcium ion binding	0.000209	***
INTERPRO, GOTERM_MF/BP/CC_DIRECT	Fatty acyl-CoA reductase, peroxisome	2.47	wat	FBgn0039620	Long-chain-fatty-acyl-CoA reductase activity, oxidation-reduction process	1.21E-06	***
			Spat	FBgn0014031	Amino transferase activity, glyoxylate catabolic process, proxisome	2.02E-02	*
			Cat	FBgn0000261	Catalase, antioxidant activity and ROS metabolic process	1.15E-02	*
			Uro	FBgn0003961	Urate oxidase activity, oxidation-reduction process, peroxisome	1.96E-02	*
			Acox57D-d	FBgn0034629	acyl-CoA dehydrogenase activity, fatty acid beta-oxidation, peroxisome	4.63E-05	***
GOTERM_MF/BP/CC_DIRECT, INTERPRO	Oxidation−reduction, haeme binding, iron ion binding, Cytochrome P450	2.2	Men	FBgn0002719	Malic oxidoreductase, determination of adult lifespan	5.16E-04	***
			Cyp6a23	FBgn0033978	Cytochrome P450, heme and iron ion binding, oxidation-reduction process	4.93E-01	NS
			Cyp4p1	FBgn0015037	Cytochrome P450, heme and iron ion binding, oxidation-reduction process	7.98E-02	.
			Cpr	FBgn0015623	NADPH-hemoprotein reductase activity, oxidation-reduction process	2.27E-04	***
			Cyt-b5-r	FBgn0000406	Heme binding, oxidoreductase activity, lipid metabolic process	2.69E-04	***
			antdh	FBgn0026268	Oxidoreductase activity, oxidation-reduction process	4.85E-02	*
			AOX1	FBgn0267408	Aldehyde oxidase, pyridoxal oxidase activity	5.82E-03	**
			Cyt-b5	FBgn0264294	Cytocrome b5-like heme-binding site	6.47E-04	***
			Cyp313a1	FBgn0038236	Cytochrome P450, heme and iron ion binding, oxidation-reduction process	1.45E-02	*
			mt: CoI	FBgn0013674	Mitochondrial Cytochrome C oxidase, heme and iron binding	7.39E-02	.
			Aldh	FBgn0012036	Aldehyde dehydrogenase, oxidation-reduction	9.39E-03	**
			Cyp4e3	FBgn0015035	Cytochrome P450, heme and iron ion binding, oxidation-reduction process	1.27E-05	***

continued

Table 4: (continued)

Annotation category	Term	Enrichment score	Gene	FlyBase gene ID	Function	*P*-value of ANOVA	Significance of ANOVA
			hgo	FBgn0040211	Homogentisate 1, 2-dioxygenase activity, oxidation-reduction process	4.08E-03	**
			Pdh	FBgn0011693	Retinol and alcohol dehydrogenase activity, oxidation-reduction process	4.75E-03	**
			Desat1	FBgn0086687	Fatty acid desaturase, oxidation-reduction process	2.55E-05	***
			Plod	FBgn0036147	Iron ion binding, oxidoreductase activity, oxidation-reduction process	6.75E-04	***
			Fad2	FBgn0029172	Fatty acid desaturase, oxidation-reduction process, lipid metabolic process	1.15E-03	**
			Gpdh	FBgn0001128	Carbohydrate metabolic process, oxidation-reduction process	6.13E-04	***
GOTERM_BP/CC/MF_DIRECT	Calcium ion channel, transmembrane transport	2.13	trpl	FBgn0005614	Calcium channel activity, calcium ion transport	2.13E-02	*
			trp	FBgn0003861	Plasma membrane cation channel, calcium channel	3.06E-09	***
GOTERM_BP/CC/MF_DIRECT	Voltage-gated potassium channel activity	2.03	Irk3	FBgn0032706	Potassium channel activity, potassium ion transport	3.88E-02	*
KEGG_PATHWAY, INTERPRO, GOTERM_BP/CC/MF_DIRECT	Fatty acid metabolism/biosynthetic process	1.93	ACC	FBgn0033246	Acetyl-CoA carboxylase activity, fatty acid biosybthetic process	3.44E-03	**
			Cyt-b5-r	FBgn0000406	Cytochrome b5-like heme binding, lipid metabolic process	6.47E-04	***
			Desat1	FBgn0086687	Fatty acid desaturase, oxidation-reduction process	2.55E-05	***
			Fad2	FBgn0029172	Fatty acid desaturase, oxidation-reduction process, lipid metabolic process	1.15E-03	**
			Acsl	FBgn0263120	Long-chain fatty acid-CoA ligase activity	1.03E-04	***
INTERPRO, GOTERM_MF_DIRECT	Carbohydrate binding	1.91	Clect27	FBgn0031629	Carbohydrate binding	4.40E-11	***
			lectin-37Db	FBgn0053533	Galactose binding	2.68E-04	***
INTERPRO, GOTERM_MF_DIRECT	Potassium ion transport, oxalate transmembrane transporter activity	1.65	Irk3	FBgn0032706	Potassium channel activity, potassium ion transport	3.88E-02	*
			Irk2	FBgn0039081	Potassium channel activity, potassium ion transport	1.96E-08	***
			Dic1	FBgn0027610	Inorganic phosphate transmembrane transporter activity	3.33E-05	***
GOTERM_MF/BP/CC_DIRECT, INTERPRO	Neurotransmitter-gated ion-channel ligand binding	1.45	Cals	FBgn0039928	Calcium ion binding	2.29E-02	*
GOTERM_MF/BP/CC_DIRECT, INTERPRO, COG_ONTOLOGY	Receptor activity, carboxylesterase, lipid metabolism	1.35	alpha-Est7	FBgn0015575	Carboxylic ester hydrolase activity, lipid storage, determination of lifespan	2.49E-04	***
			ACC	FBgn0033246	Acetyl-CoA carboxylase activity, fatty acid biosybthetic process	3.44E-03	**
			Gs2	FBgn0001145	Glutamate catabolic process, neurotransmitter receptor metabolic process	1.46E-03	**
			Est-6	FBgn0000592	Carboxylic ester hydrolase activity	2.93E-02	*
			AcCoAS	FBgn0012034	Acetyl-CoA ligase activity	3.35E-04	***
GOTERM_MF/BP/CC_DIRECT, INTERPRO	Neurotransmitter transporter	1.32	NAAT1	FBgn0029762	Neurotransmitter transporter activity	2.06E-08	***
			Eaat1	FBgn0026439	Glutamate: sodium symporter activity, determination of adult lifespan	2.06E-02	*

Genes that were significantly down-regulated in these data in HPLC compared to LPHC were compared to a background list of genes that were expressed and detected in these data. Analyses of variance were carried out on expression between all samples in this study (F0–F3 matched and mismatched) with *P*-values and statistical significances listed.

direct exposure, some studies searching for evidence of true transgenerational inheritance are declining to assay the F1 generation entirely [79] due to the fact that transmission to the F1 generation can be indicative of both parental effects and programming [80]. Thus, here we describe our findings from the F1 and F2 generations as multigenerational inheritance, because the gene expression changes induced by the environment in the parental generation revert to F0 levels after the F2 generation.

The current NCD epidemic is aetiologically very complex, but it is thought to be mediated, in part, by developmental aberrations arising from the inheritance of altered epigenetic marks [2, 5]. Many metabolic phenotypes and gene expression differences are linked to differential epigenetic marks that are nutritionally induced. For example, a protein-restricted diet during pregnancy causes hypomethylation of the hepatic PPARα and glucocorticoid receptor genes in rats and promotes the same hypomethylation in the F1 and F2 offspring of F0 rats fed a protein-restricted diet during pregnancy, despite the nutritional challenge being only in the F0 generation [27]. Others have reported evidence of embryonic environmental exposure influencing the phenotype of the F1 generation in species as diverse as humans, rats, chordate fish, Daphnia and isopods [59, 60, 81–84] as well as, specifically, maternal nutrition exerting effects on the F1 phenotype [59, 60]. Such research strongly implies that epigenetic effects could be the key to understanding the current epidemic of overweight and obese, and associated metabolic syndromes, particularly if nutrition in the F0 generation can induce a PAR to nutrition, as we hypothesize is occurring here. Interestingly, comprehensive studies using animal models that investigated the effect of both protein restricted and energy-rich diets during pregnancy on the phenotype of the offspring showed that offspring born to dams fed these different diets exhibited persistent metabolic changes, similar to those observed in human metabolic disease such as obesity, insulin resistance and hypertension [15], indicating an element of developmental programming and a possible PAR. These findings imply that both famine (protein restriction) and energy-rich diets, when mismatched back to adequate nutrition, are similarly detrimental to the metabolic health of offspring and that it is possibly the mismatch itself between inadequate nutrition and proper nutrition which is leading to metabolic disease phenotypes. This highlights the fact that epigenetic mechanisms play a highly complex role in human obesity and metabolic pathways [15, 85–87].

In addition to the striking expression level changes observed in genes that have GO terms classified as having roles in epigenetic processes, one major source of change that we observed in these data are genes involved in oxidative stress. Malnutrition or excess of particular nutrients can cause oxidative damage [88]. For example, hyperglycaemia, which is an excess of sugar in the blood and one of the hallmarks of diabetes, is linked to a diet that is rich in carbohydrates and fat [89, 90]. continuedAn accumulation of sugar can lead to tissue damage, and this can be maintained because of metabolic memory [35], which itself may induce epigenetic changes and altered gene expression [35, 91, 92]. Given that the increased carbohydrate intake can induce oxidative stress as reflected by increased ROS generation [51, 52], our results are consistent with the observation that high-carbohydrate diets are implicated in increased oxidative and metabolic stress [e.g. [49] and that the genome may be responding adaptively to dietary stressors]. We know that oxidative stress responses are often under epigenetic control [55] and also, that maternal nutritional deficiency in pregnancy can lead to altered methylation and increased oxidative DNA damage in the brains of adult offspring [93], which, as well as being directly influenced by nutrition, may predispose to neurological disorders in later life.

Consistent with, and leading on from this observation, these data display a decrease in the expression of genes involved in neurotransmission and neurodevelopment when exposed to an HPLC diet. There is strong evidence linking oxidative stress to neurodegeneration and neurodegenerative disease, such as Alzheimer's disease [94]. In addition, it is also clear that an increase in the production of ROS, induced by environmental factors, can increase the risk of a multitude of neurodegenerative diseases [95]. Thus, it stands to reason that, in these data, nutrition may be impacting on the production of ROS and the expression of genes involved in ROS pathways and those involved in neurotransmission and neurodegeneration.

In addition to the DOHaD hypothesis, there are also free radical early-life theories, which link environmental agents (e.g. diet and heavy metals) with perturbations of gene regulation and expression (e.g. in the *APP* gene) and the onset of, for example Alzheimer's disease [96]. Free radical early-life theories also link the necessity for oxygen in histone demethylase action to epigenetic processes in development [97]. These theories are supported by the observation that nutrition during pregnancy can induce epigenetic changes that result in altered nervous system development [98] and also offspring cerebral function [99]. Further, nutrient availability during the pre and postnatal periods can lead to long-lasting changes in neuron development [100] as well as influence the development of psychopathological behaviour [101]. This is because nutritional deficit may lead to altered brain development [102], possibly via epigenetic factors that can lead to changes in brain structure and function [103]. Micronutrient availability can heavily influence neurotransmission, due to the fact that the function of the brain is inherently related to its metabolism of nutrients [103] in the form of vitamins and minerals that function as co-enzymes in neurotransmission and neurotransmitter metabolism. Given that the gene expression data presented here display significant changes in gene expression in pathways relevant to neurotransmission, these data are supportive of these linkages.

Thus, through our data, we hypothesize that the genome-wide changes we observe in genes involved in epigenetic pathways could be responsible for the gene expression changes in other, broad, biological process seen in response to diet. The intermediate maintenance of these gene expression changes, even when the HPLC diet is removed, suggests a PAR to diet; the biology of the mismatched flies is programmed to respond to a particular diet, and is responding adaptively, with altered gene expression in the absence of HPLC, albeit at a slightly lower level. The complete reversion of this in the F3 generation suggests an element of phenotypic rescue, implying that altered nutrition did not affect the germ line and that the gene expression changes are not fixed transgenerationally and thus may have the capacity to be corrected over time.

To date, while the effects of dietary manipulation on fecundity and lifespan in *Drosophila* have been reported [30, 104–106], none of the studies have assayed the whole-genome gene expression in response to diet. This study contributes to our understanding of the myriad ways in which nutrition can influence gene expression, phenotypes and future health outcomes, with relevance to the DOHaD hypothesis and the current NCD epidemic. While our study demonstrates multigenerational inheritance of gene expression values, rather than transgenerational, it is worth noting that the phenotypic effects of gene expression changes, rather than the gene expression itself, can

Table 5: A functional annotation comparison of up and downregulated genes at each generation

GO category	GO term	F0				F1				F2				F3			
		$n = 1074$		$n = 1867$		$n = 541$		$n = 1684$		$n = 17$		$n = 22$		$n = 1893$		$n = 2720$	
		↑ expression in HPLC		↓ expressison in HPLC		↓ expression in MM		↑ expression in MM		↓ expression in MM		↑ expression in MM		↓ expression in MM		↑ expression in MM	
Biological process		**# genes**	**% of total**	**# genes**	**% of total**	**# genes**	**% of total**	**# genes**	**% of total**	**# genes**	**% of total**	**# genes**	**% of total**	**# genes**	**% of total**	**# genes**	**% of total**
Biological adhesion	GO: 0022610	3	0.30	34	1.90	1	0.20	29	1.90	1	7.10			7	0.40	35	1.50
Biological regulation	GO: 0065007	80	7.60	127	7.00	25	5.10	109	7.20			1	4.50	143	7.50	175	7.40
Cellular component organization or biogenesis	GO: 0071840	93	8.90	63	3.50	36	7.30	51	3.40			1	4.50	176	9.20	98	4.10
Cellular process	GO: 0009987	370	35.30	475	26.30	164	33.50	383	25.50	2	14.30	4	18.20	696	36.30	602	25.50
Developmental process	GO: 0032502	37	3.50	104	5.80	11	2.20	89	5.90			2	9.10	70	3.60	147	6.20
Immune system process	GO: 0002376	10	1.00	26	1.40	5	1.00	25	1.70					16	0.80	33	1.40
Localization	GO: 0051179	78	7.40	139	7.70	25	5.10	130	8.60	2	14.30	2	9.10	151	7.90	194	8.20
Locomotion	GO: 0040011	4	0.40	1	0.10	2	0.40	1	0.10					6	0.30	1	0.00
Metabolic process	GO: 0008152	383	36.50	393	21.80	179	36.50	328	21.80	5	35.70	5	22.70	708	36.90	508	21.50
Multicellular organismal process	GO: 0032501	18	1.70	127	7.00	6	1.20	90	6.00			2	9.10	28	1.50	162	6.90
Reproduction	GO: 0000003	16	1.50	30	1.70	9	1.80	24	1.60			1	4.50	28	1.50	36	1.50
Response to stimulus	GO: 0050896	75	7.20	124	6.90	33	6.70	106	7.00			1	4.50	140	7.30	166	7.00
Rhythmic process	GO: 0048511			14	0.80	1	0.20	13	0.90			1	4.50	1	0.10	14	0.60
Protein class		**# genes**	**% of total**	**# genes**	**% of total**	**# genes**	**% of total**	**# genes**	**% of total**	**# genes**	**% of total**	**# genes**	**% of total**	**# genes**	**% of total**	**# genes**	**% of total**
Calcium-binding protein	PC00060	4	0.40	27	1.50	1	0.20	14	0.90					17	0.90	35	1.50
Cell adhesion molecule	PC00069	2	0.20	24	1.30			16	1.10					5	0.30	30	1.30
Cell junction protein	PC00070	3	0.30%	8	0.40			10	0.70					5	0.30	14	0.60
Chaperone	PC00072	8	0.80%	3	0.20	2	0.40%	3	0.20					14	0.70	7	0.30
Cytoskeletal protein	PC00085	41	3.90%	44	2.40	17	3.50%	38	2.50					59	3.10	59	2.50
Defense/immunity protein	PC00090	2	0.20%	8	0.40	1	0.20%	7	0.50					7	0.40	10	0.40
Enzyme modulator	PC00095	42	4.00%	59	3.30	14	2.90%	51	3.40			1	4.50%	101	5.30	72	3.00
Extracellular matrix protein	PC00102			22	1.20			16	1.10					3	0.20	26	1.10
Hydrolase	PC00121	73	7.00%	144	8.00	30	6.10%	131	8.70	4	28.60%	2	9.10%	142	7.40	181	7.70
Isomerase	PC00135	5	0.50%	5	0.30			8	0.50					13	0.70	7	0.30
Ligase	PC00142	24	2.30%	20	1.10	8	1.60%	15	1.00	1	7.10%	1	4.50%	48	2.50	25	1.10
Lyase	PC00144	4	0.40%	28	1.60			20	1.30					15	0.80	25	1.10
Membrane traffic protein	PC00150	6	0.60%	16	0.90			15	1.00					14	0.70	21	0.90
Nucleic acid binding	PC00171	164	15.60%	72	4.00	88	18.00%	60	4.00					296	15.40	134	5.70
Oxidoreductase	PC00176	21	2.00%	94	5.20	12	2.40%	67	4.50					43	2.20	96	4.10
Receptor	PC00197	12	1.10%	81	4.50	5	1.00%	64	4.30	1	7.10%			28	1.50	107	4.50
Signalling molecule	PC00207	12	1.10%	56	3.10	3	0.60%	49	3.30			1	4.50%	24	1.30	72	3.00

continued

Table 5: (continued)

GO category	GO term	F0				F1				F2				F3			
		$n = 1074$		$n = 1867$		$n = 541$		$n = 1684$		$n = 17$		$n = 22$		$n = 1893$		$n = 2720$	
		↑ expression in HPLC		↓ expressison in HPLC		↓ expression in MM		↑ expression in MM		↓ expression in MM		↑ expression in MM		↓ expression in MM		↑ expression in MM	
Storage protein	PC00210	3	0.30%	7	0.40	2	0.40%	4	0.30					5	0.30	8	0.30
Structural protein	PC00211	2	0.20	2	0.10	1	0.20	3	0.20					7	0.40	3	0.10
Transcription factor	PC00218	81	7.70	68	3.80	33	6.70%	59	3.90					130	6.80	117	5.00
Transfer/carrier protein	PC00219	13	1.20%	36	2.00	1	0.20%	31	2.10					28	1.50	51	2.20
Transferase	PC00220	62	5.90%	56	3.10	25	5.10%	49	3.30			1	4.50%	131	6.80	66	2.80
Transmembrane receptor regulatory/adaptor protein	PC00226	1	0.10%	6	0.30	8	1.60%	4	0.30					3	0.20	5	0.20
Transporter	PC00227	28	2.70%	140	7.80			108	7.20					47	2.50	168	7.10
Viral protein	PC00237			1	0.10												
Molecular function		**# genes**	**% of total**	**# genes**	**% of total**	**# genes**	**% of total**	**# genes**	**% of total**	**# genes**	**% of total**	**# genes**	**% of total**	**# genes**	**% of total**	**# genes**	**% of total**
Antioxidant activity	GO: 0016209	2	0.20	9	0.50	3	0.60	7	0.50					5	0.30	12	0.50
Binding	GO: 0005488	258	24.60	240	13.30	117	23.90	201	13.40	1	7.10%	1	4.50%	455	23.70	349	14.80
Catalytic activity	GO: 0003824	265	25.30	412	22.80	123	25.10	352	23.40%	5	35.70%	4	18.20%	524	27.30	483	20.40
Channel regulator activity	GO: 0016247															1	0.00
Receptor activity	GO: 0004872	8	0.80	54	3.00	5	1.00	47	3.10	1	7.10			18	0.90	74	3.10
Signal transducer activity	GO: 0004871	3	0.30	12	0.70	1	0.20	8	0.50					7	0.40	19	0.80
Structural molecule activity	GO: 0005198	22	2.10	64	3.50	6	1.20	44	2.90					45	2.30	87	3.70
Translation regulator activity	GO: 0045182	2	0.20	1	0.10	1	0.20	115	7.60					9	0.50	3	0.10
Transporter activity	GO: 0005215	34	3.20	149	8.30	13	2.70			1	7.10	1	4.50	58	3.00	177	7.50

Up- and down-regulated genes at each generation were submitted to PANTHER for an analysis of their annotated function. Genes were put into gene ontology (GO) classes, and the number of genes from each of the up- and down-regulated gene list in each class is reported. F0 compares the number of genes in each GO class that are up-regulated or down-regulated in HPLC vs. LPHC. For each subsequent generation (F1–F3), the number of genes in each class is reported as those that are up- or down-regulated in mismatched (MM) flies vs. matched (M) flies, at each generation. The percentages reported are the percentage of gene hits against the total number of genes identified in each list submitted for gene functional classification.

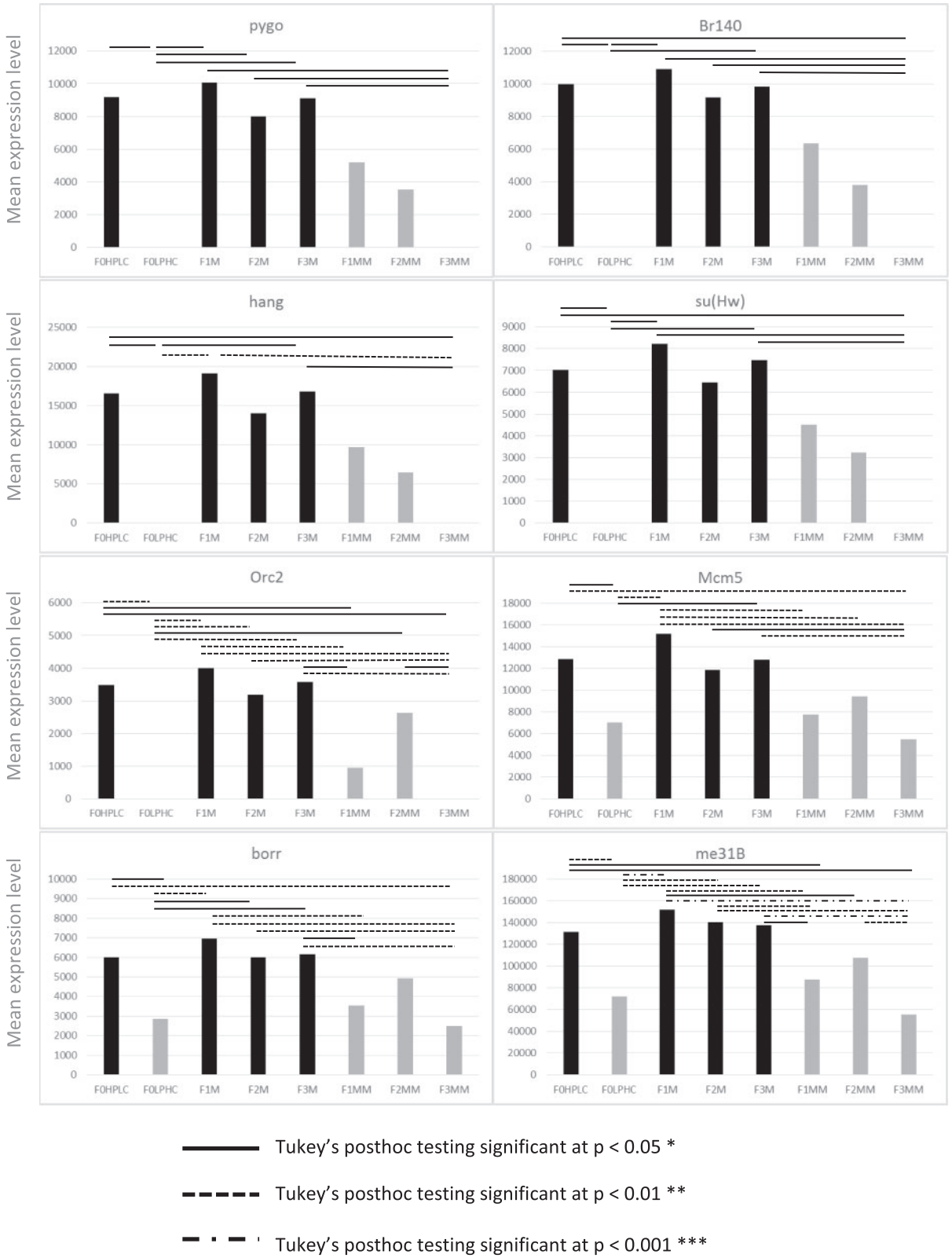

Figure 2: Indicative graphs of gene expression of genes up-regulated in the F0 generation, with significances as determined by ANOVA, between F0 and F3 generations on matched and mismatched diets. Pairwise comparisons by Tukey's *post hoc* testing indicated by solid and dashed lines, as described in the key. *Y* axis denotes mean expression level from transcriptomic experiments, and *X* axis denotes the dietary condition as per Figure. 1. Note differing *Y* axis scales. Gene names are stated as per their FlyBase gene symbol IDs.

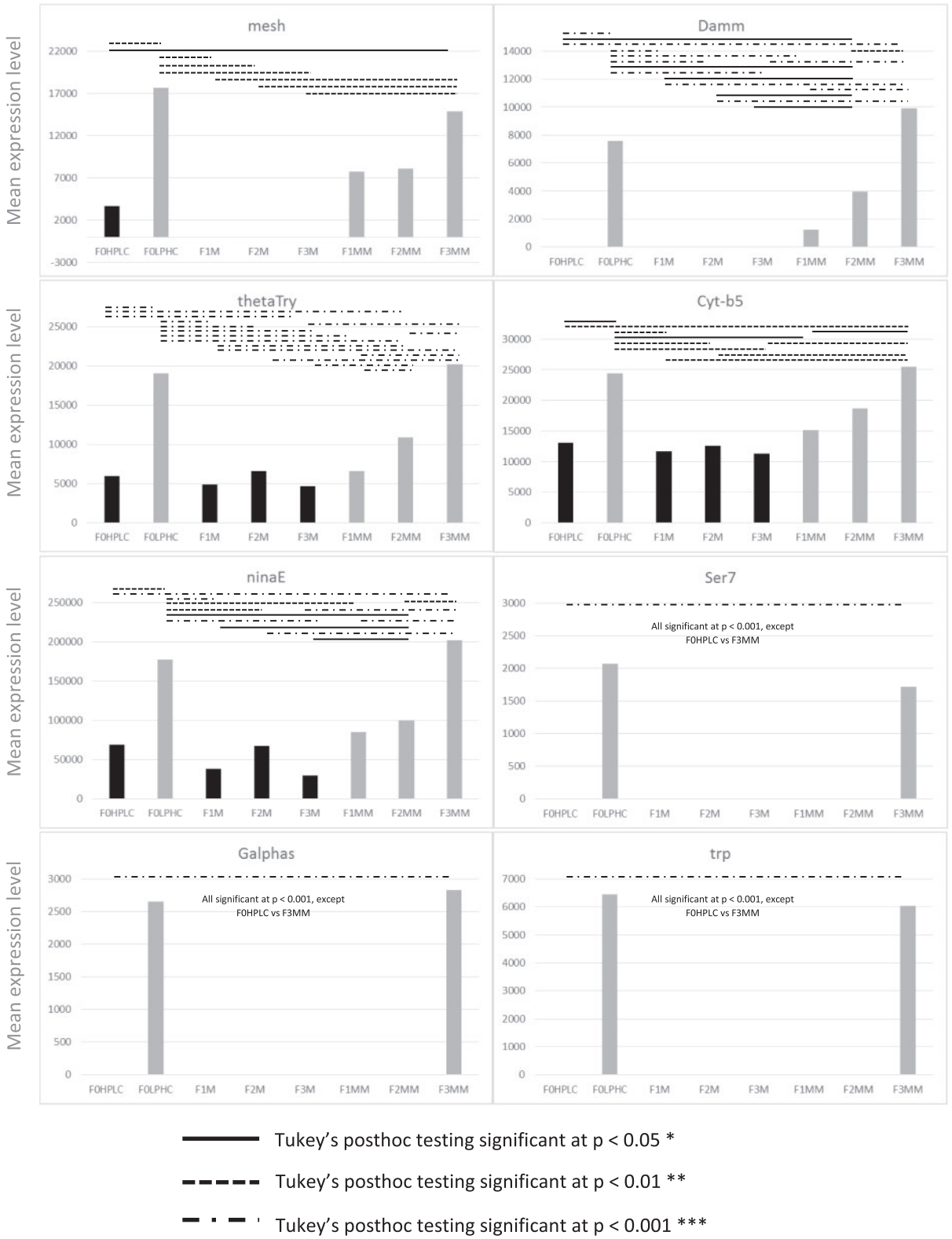

Figure 3: Indicative graphs of gene expression of genes down-regulated in the F0 generation, with significances as determined by ANOVA, between F0 and F3 generations on matched and mismatched diets. Pairwise comparisons by Tukey's *post hoc* testing indicated by solid and dashed lines, as described in the key. *Y* axis denotes mean expression level from transcriptomic experiments, and *X* axis denotes the dietary condition as per Figure. 1. Note differing *Y* axis scales. Gene names are stated as per their FlyBase gene symbol IDs.

persist and show multigenerational, and potentially transgenerational, inheritance. For example, a low-protein diet given to *Drosophila* can increase H3K27me3 through up-regulation of the Enhancer of zeste (E(z)) protein, a protein that is a catalytic component of the Polycomb Repressive Complex 2 methyltransferase. Interestingly, while the up-regulation of the (E)z protein was not detected in the F2 generation, the associated increase in methylation H3K27me3 (a specific chemical modification – trimethylation – of histone H3 at the lysine 27 residue) was in fact detected in the F2 generation [79] and the co-ordinated effect on longevity was also present through to the F2 generation. This suggests that while the gene expression and protein level is not inherited *per se*, the effects and/or functions of those genes possibly could be. It is possible that a phenomenon such as this may be present in these data; a permissive state may be achieved whereby we might not detect gene expression changes inherited to F3 and beyond, but we may see associated genomic conformational or phenotypic changes in F3 and beyond. Further functional studies based on dietary manipulation are required to confirm this. In particular, it will be pertinent to prove causality between an epigenetic alteration and a change in regulation of genes involved in the traits we observe. To do so, we suggest a combination of phenotypic measures such as assessing lifespan, oxidative stress resistance, and immunity, as well as exploiting mutant *Drosophila* strains for genes of interest, to assess the effect of epigenetic alterations on downstream gene expression and associated phenotypes. Further functional studies are especially pertinent due to the wealth of evidence demonstrating that high-protein diets in *Drosophila* can alter *Drosophila* development and fitness, particularly ovary development and reproductive output [107–110], heat stress tolerance [110, 111], body composition [109] and egg-to-adult viability [111]. The different caloric content between the HPLC and LPHC diets cannot be discounted as a confounding factor; however, the wealth of evidence pertaining to the epigenetic effects of high-protein diets that support these data implies that the gene expression changes detected in these data are driven by the protein/carbohydrate content of the food, rather than a caloric difference.

Thus, here we have identified characteristic suites of genes that are responsive to altered nutrition and the maintenance of altered gene expression upon removal of the nutritional challenge in multiple generations. This has broad implications for our understanding of DOHaD, the PAR hypothesis and the NCD epidemic and will be vital in directing future functional research in this area.

Acknowledgements

Stocks obtained from the Bloomington Drosophila Stock Center (NIH P40OD018537) were used in this study. Under New Zealand law, *Drosophila* research is waived from requiring ethical approval, therefore none were needed for this study. *Drosophila melanogaster* stocks were imported under the HSNO Approval number GMC001092. All *Drosophila* work was carried out in accordance with the regulations of the HSNO Act 1996, as described in Morgan, 2012. We are grateful to Professor Sir Peter Gluckman for his input into, and discussions around, this subject; Matthew Walters for technical assistance; and three anonymous referees, whose comments improved this manuscript. A.J.O. would like to thank Professor Martin Kennedy for hosting the latter stages of this work.

Data Availability

All data supporting this work is included in the supplementary material.

Funding

This work was supported by a Gravida grant (MP04) to P.K.D.

Supplementary data

Supplementary data are available at *EnvEpig* online.

Conflict of interest statement. None declared.

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