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Article

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Changes in animal nutrition, particularly essential dietary components, alter global gene expression patterns. Our goal is to identify molecular markers that serve as early indicators of the quality of insect culture media. Markers of deficient culture media will increase the efficiency of developing optimal systems for mass rearing beneficial insects and some pest species because decisions on culture media quality can be made without waiting through one or several life cycles. The objective of our current study is to discover molecular markers of essential dietary lipid deficiency in the oriental fruit fly, Bactrocera dorsalis. We reared groups of fruit flies separately on media either devoid of or supplemented with wheat germ oil (WGO) and analyzed gene expression in third instar larvae and F_1 eggs using 2D electrophoresis. Gel densitometry revealed significant changes in expression levels of genes encoding eight proteins in larvae and 22 proteins in eggs. We identified these proteins by using mass spectrometry (MALDI TOF/TOF) and bioinformatic analyses of the protein sequences. Among these, we identified one gene encoding the receptor of activated C Kinase 1 (RACK1) that increased in expression by 6.8-fold in eggs from adults that were reared as larvae on media

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supplemented with WGO. RACK1 is an essential component of at least three intracellular signal transduction pathways, making it a good molecular marker candidate of lipid deficiency in fruit flies and possibly many other insect species. © 2010 Wiley Periodicals, Inc.

Keywords: wheat germ oil; fruit fly diet; *Bactrocera dorsalis*; proteomics

INTRODUCTION

Sterile insect technique used in the control of the oriental fruit fly, *Bactrocera dorsalis*, requires continuous mass rearing (Parker, 2005). As seen in the highly successful experience with screwworm eradication project, formulation of an economically and biologically effective culture system for screwworm larvae required decades of research. The lengthy research program is due in part to the length of the insect life cycle and the need to conduct nutritional research over several consecutive generations. It may require an entire year to evaluate a single change in a culture medium. The efficiency of the process could be improved by the discovery of molecular markers of nutritional deficiency that are present in the egg or early larval stage of development, which could be used to make decisions on media quality without waiting through one or more insect life cycles. Diet influences gene expression in insects (Yocum et al., 2006). Our goal is to identify early molecular markers of essential nutritional deficiency (Coudron et al., 2006). The intent is to apply those early indicators to direct diet formulation for improving the fitness and reducing the cost of rearing the oriental fruit fly.

The addition of wheat germ oil (WGO) to a liquid fruit fly diet enhanced rearing efficiency by significantly improving several parameters of biological fitness. Larval development, pupal recovery, percentage of adult fliers, egg production, and hatch were improved, most likely because WGO provides substantial quantities of essential polyunsaturated fatty acids (Dadd, 1985; Chang and Vargas, 2007; Chang, 2009). The positive nutritional influence of WGO or other sources of polyunsaturated fatty acids in insect diets has been thoroughly documented (Dadd, 1985). However, it is not clear how dietary WGO benefits insects. One mechanism of dietary WGO action in fruit fly larvae is its influence on gene expression in mature adults (Chang et al., 2010). Dietary WGO led to enhanced expression of some genes and to decreased expression of others.

Given evidence that dietary WGO influenced gene expression in adult fruit flies, we posed the hypothesis that dietary WGO would also influence gene expression in larvae and subsequent F_1 egg stages. In this study, we report on the outcomes of experiments designed to test our hypothesis.

MATERIAL AND METHODS

Insects and Sample Collection

Newly collected eggs (<6 hr) of oriental fruit fly, *B. dorsalis* (Hendel) were provided by the Tropical Crop and Commodity Protection Research Unit of the USDA's Agricultural Research Service (ARS) in Honolulu, Hawaii and maintained as previously described (Chang et al., 2010). *B. dorsalis* larvae were reared on a liquid diet devoid of, or separately supplemented with, WGO (0.66%, v:v) (Chang and Vargas, 2007). Adults from both larval diets were maintained on a mixture of sugar:protein hydrolysate

(3:1, wt:wt) (Chang et al., 2004). Samples included third instar larvae and F_1 eggs laid by adults that developed from larvae reared on each of these diets.

Sample Preparation

Samples were homogenized 3 × 15 sec on ice in 10 mM Tris–HCl (pH7.0) containing protease inhibitors (final dilution = 1:100; Sigma, St. Louis, MO, ♯ P8340 for Mammalian Cell and Tissue Extracts) using a Tissue Master (Omni Intl., Marietta, GA). Homogenates were centrifuged twice at 15,294 × *g* for 15 min at 4°C. The middle layer of homogenized samples was retained and transferred to new vials on ice for immediate use. Three independent biological replicates were processed for each treatment.

2D-Electrophoresis

Electrophoresis and mass spectrometric protocols followed published procedures (Stanley et al., 2008). Protein amounts were determined using the Pierce Micro BCA Protein Assay Kit, using BSA as a quantitative standard (Rockford, IL). Protein samples were prepared for iso-electric focusing (IEF), as previously described (Chang et al., 2010). IEF was performed with a Protean IEF cell system (Bio-Rad, Hercules, CA) using the standard protocol and a pre-set linear volt ramp program (8,000 V and 50 μA/strip max., 35,000 vH).

For the second dimension, the IPG strips were prepared and subjected to SDS-PAGE using the Criterion Cell system (Bio-Rad ♯165–6001) with a precast gel (8–16% Tris–HCl for egg samples and 4–15% Tris–HCl for larval samples; Bio-Rad). Gels were stained with Coommassie Blue G-250 (BioSafe Stain; Bio-Rad) and analyzed using Delta 2D software (Decodon GmbH, Greifswald, Germany). Protein spots with densities significantly different between treatments (Students' *t*-test, $P < 0.05$) were removed using a 1.5-mm spot picker (The Gel Company, San Francisco, CA) and stored at −80°C. One gel was run for each independent biological replicate.

MS/MS Analysis

Proteins were digested with trypsin and prepared for MS/MS analysis as previously described (Stanley et al., 2008). A portion of each protein was mixed with alpha-cyano-4-hydroxycinnamic acid matrix and applied to the MALDI target and analyzed. The resulting sequence data, combined with observed MW and pI values, were used to establish protein identities. Sequences generated from these analyses were used to interrogate NCBI-BLAST for protein matches (using the PAM30 matrix and searching within "Metazoa"). E-values and frequency of matches to a specific protein were the primary criteria for these determinations.

Quantitative Real-Time PCR

Total RNA was isolated from fly tissues using the TRIzol Reagent (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. RNA concentrations were measured at 260 nm on a Nanodrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA). The extracted total RNA was treated with RNase-free DNase I (Promega, Madison, WI, ♯ M6101). The first-strand cDNA was synthesized from 3 μg total RNA using Oligo(dT) primers and superscript II reverse transcriptase (Invitrogen) according to the manufacturer's protocol.

Real-time PCR (RT-PCR) amplification and analysis were performed on the Eppendorf Master Cycle Rep Realplex 4S and analyzed with the Realplex software (Eppendorf, Hauppauge, NY). RT-PCR was carried out using Absolute QPCR SYBR Green kit (Thermo Scientific, Pittsburg, PA) according to the manufacturer's procedure in 20 μl reaction volumes. The RT-PCR program used was: hold at 95°C for 15 min followed by 50 cycles at 95°C for 15 sec, 52°C for 30 sec, then 72°C for 30 sec. The specificity of the SYBR Green PCR signal was further confirmed by melting curve analysis. The mRNA expression was quantified using the comparative CT (cross threshold, the PCR cycle number that crosses the signal threshold) method (Livak and Schmittgen, 2001). The cDNA was amplified using gene-specific primers. The primers for protein spot E17 were 5′-CGACCCTTTATCGATGTGAACTACATGG-′3 and 5′-ATGTTGGCTGGGTCGCG-′3; primers for protein spot E20 were 5′-GCTACAAGGCAGCAAGTTC-′3 and 5′-GAGAACTTCAAGCTGAAGCACTA-′3. To normalize the cDNA, the primers for the housekeeping gene actin (5-TCCATCATGAAGTGCGACGT-′3 and 5′-AGAAG-CACTTGCGGTGGACGA-′3) were also used to amplify cDNA from the samples.

The values generated for quantitative real-time PCR represent the mean and standard error of the ratio of the quantity of selected mRNA for treatments with WGO/without WGO, for six independent replications. All the data are presented as mean ± standard errors. Data were analyzed by Students' *t*-test utilizing NCSS 2007 (Kaysville, UT).

RESULTS

Representative 2D protein gels are presented in Figure 1 for third instar larvae reared on each of the diets and in Figure 2 for F_1 eggs laid by adults from the larvae reared on each of the diets. Quantitative gel analysis detected approximately 380 spots/gel from larval preparations for each treatment and over 800 proteins from egg preparations. Gel densitometry revealed changes, by 2-fold or more, in expression of genes encoding eight larval proteins and 43 egg proteins. These spots were selected for digestion and analysis by MALDI-TOF MS/MS. We identified eight larval proteins and 23 egg proteins by in silico analysis.

Media supplemented with WGO led to decreased expression of eight genes in larvae. Genes encoding larval proteins L-10, L-16, L-26, L-22, and L-21 were downregulated by approximately 50% and the remaining three genes were downregulated by 20–40% (Tables 1 and 2). Protein functions affected included those involved in protein degradation and folding, signal transduction, and cell protection.

For F_1 eggs, larval media supplemented with WGO led to up- and down-regulation of gene expression. WGO influenced the expression of genes encoding proteins involved with protein metabolism, structure and function, signal transduction, cell protection, DNA replication and repair, energy and metabolism, intracellular signaling, and lipid transport (Tables 3 and 4). Genes encoding proteins E-06, E-08, E-68, E-98, and E-93 were upregulated by twofold or more. WGO also led to downregulation of several genes.

We designed primers to genes corresponding to two proteins (E-17 and E-20; Fig. 3) that changed in expression on analysis of 2-D gels. Genes were selected on the basis of their overall expression level and efficiency of design. We used these primers to confirm parallel changes in protein spot densities and mRNA expression. For both

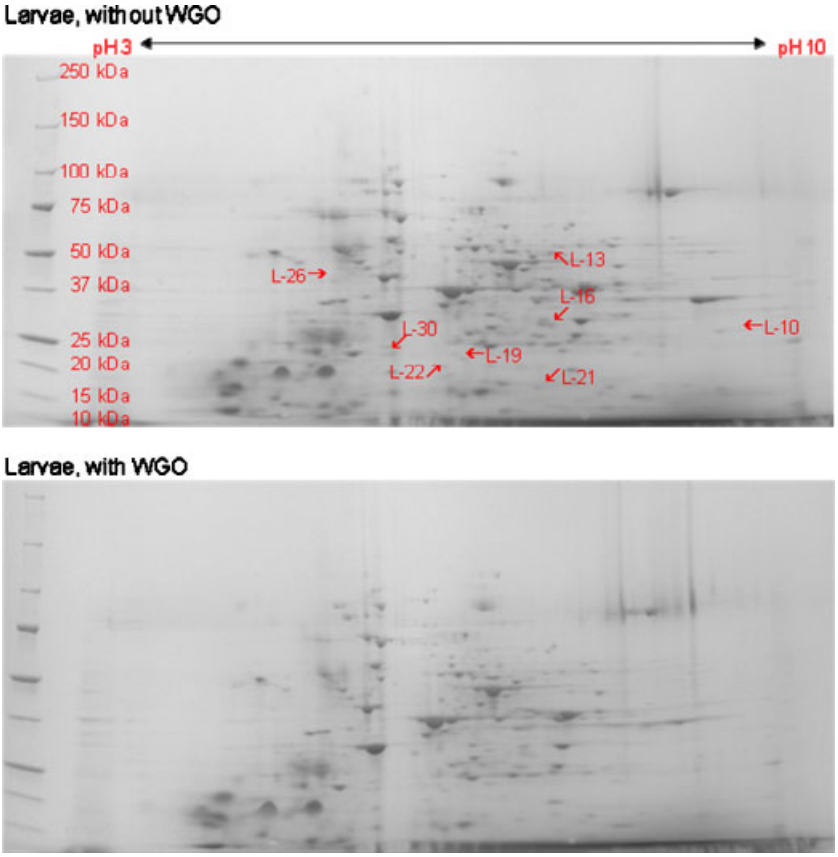

Figure 1. Representative 2D gels showing the influence of dietary WGO on protein expression in larvae reared on diet with and without WGO. The labeled spots in this image were selected for extraction and MS/MS analysis. WGO, wheat germ oil.

proteins, mRNA and protein levels were lower in eggs oviposited by adults that were reared on diet supplemented with WGO during the larval stage.

DISCUSSION

The data reported in this paper strongly support our hypothesis that supplementing larval fruit fly culture medium with WGO influences gene expression, recorded by changes in whole-organism cellular protein profiles, in third instar larvae and subsequent F_1 eggs. The influence of dietary WGO, which we attribute to essential polyunsaturated fatty acids, on gene expression can be registered without first separating the genders, even in juvenile insects. We did not determine the gender of larvae and F_1 eggs and the changes in gene expression we recorded represent up- and downregulating influences for unknown proportions of males and females. This is a valuable finding because determining gender can be a difficult and resource consuming exercise. Our discovery that changes in nutritional quality influence gene expression in egg and larval stages supports efforts to find molecular markers of nutritional deficiency at early developmental stages.

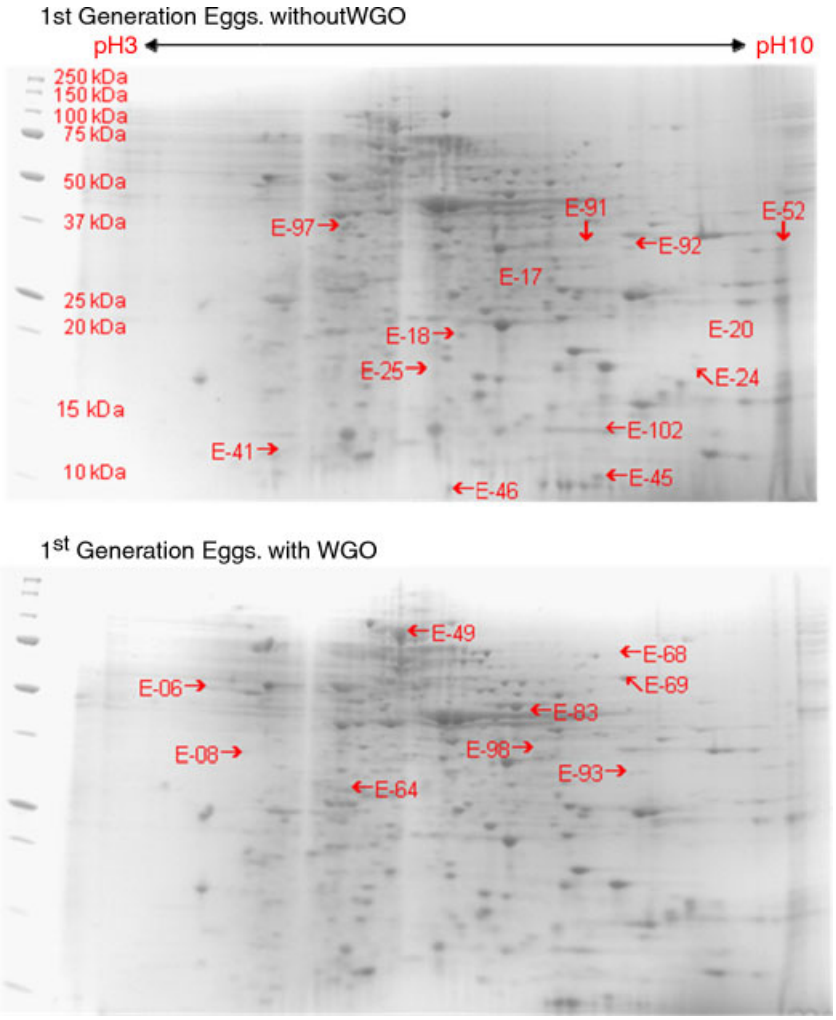

Figure 2. Representative 2D gels showing the influence of dietary WGO on protein expression in eggs oviposited by adults from larvae reared on diet with and without WGO. The labeled spots in this image were selected for extraction and MS/MS analysis. WGO, wheat germ oil.

WGO influenced the expression of more genes during the F_1 egg stage than the third instar larvae taken directly from the WGO-supplemented medium. It also exerted larger influences on gene expression in eggs than in larvae. WGO supplementation in the larval diet resulted in downregulation of several genes in the third instar larvae. We speculate that lower expression in lipid-rich larvae relative to larvae taken from the lipid-deficient medium indicates a greater need for these eight proteins, and possibly others, in larvae reared on lipid-deficient diets. We now turn to discussion of a few selected genes, those that changed in expression by at least 2-fold.

For larvae, only L-10 changed in expression level by twofold or more. This protein corresponds to protein M-57 (Male 57; declined by 20%) in our previous paper (Chang et al., 2010). L-10 (and M-57) is a member of the 70-kDa heat shock proteins

Table 1. Proteins From Bactrocera dorsalis Larval Homogenates Identified Using Mascot

Spot no.	*Putative protein with accession no. (species)*	*Calculated mass*[a]	*Calculated pI*[a]	*MOWSE protein score*[b]	*Total ion score*[c]	*No. peptides*[d]	*% Protein coverage*[e]	*E-value*[f]	*Peptide sequence (with highest ion score)*[g]
L-10	Heat shock-like protein, similar to heat shock 70 kDa proteins *Ceratitis capitata* gi|662802	71,435	5.36	333	295	32	52	8.8e−28	TTPSYVAFTETER (67)
L-13	Glutathione reductase family member *Musca domestica* gi|1848294	53,896	6.34	154	120	18	54	7e−10	ILLNTVGIHPTTAEEFTR (120)
L-16	20S proteasome subunit alpha1 *Drosophila melanogaster* gi|4929308	27,460	7.55	131	104	18	73	1.4e−07	HITIFSPEGR (65)
L-19	GF19402 *Drosophila ananassae* gi|194768002	21,952	5.52	119	97	6	30	2.1e−06	DYGVLDEETGIPFR (97)
L-21	GJ23442 *Drosophila virilis* gi|195389180	24,200	8.99	449	419	15	59	2.2e−39	KYGVPEEEIFQTADLFER (141)
L-22	GL17374 *Drosophila persimilis* gi|195153795	20,508	5.97	125	115	6	44	5.6e−07	RFEEQGVEFVK (69)
L-26	Heat shock-like protein, similar to heat shock 70 kDa proteins *C. capitata* gi|662802	71,435	5.36	498	381	45	70	2.8e−44	SAIHDIVLVGGSTR (88)
L-30	GJ22817 *Drosophila virilis* gi|195391538	26,477	7.68	138	122	11	38	2.8e−08	QYSINDLPVGR (72)

[a]As reported in Mascot search results.
[b]MOWSE protein scores >75 are significant ($P<0.05$) for the Metazoa search.
[c]Ion scores >45 are significant ($P<0.05$).
[d]The number of peptide sequences identified by Mascot that contributed to the MOWSE Score.
[e]The percent of the protein sequence that is accounted for by the matching peptide sequences.
[f]E-value generated by Mascot.
[g]Sequences of the peptides with the highest ion scores. The individual ion scores are shown in brackets.

Table 2. Relative Changes in Gene Expression and Function of Proteins Identified by MS/MS From Larvae Fed on Diet With or Without Wheat Germ Oil (+WGO or –WGO, respectively)

Spot no.	*+WGO/−WGO ratio*	*Protein function*
Protein structure, function and degradation		
L-10	0.47	Involved in protein folding and signal transduction
L-16	0.56	Involved in nonlysosomal protein degradation
L-26	0.55	Involved in protein folding and signal transduction
Cell protection		
L-13	0.57	Catalyzes the reduction of oxidized glutathione
L-19	0.78	Peroxiredoxin: thiol-specific antioxidant protects cells by reducing hydrogen peroxide, peroxynitrite, and organic hydroperoxides
L-22	0.55	Glyoxalase/Bleomycin resistance protein/Dioxygenase: detoxifies endogenous toxins
L-30	0.69	Peroxiredoxin: reducing hydrogen peroxide, peroxynitrite, and organic hydroperoxides
Signal transduction and cytoskeleton		
L-21	0.54	Actin-binding domain: found in cytoskeletal and signal transduction proteins

(HSP), which are ubiquitously expressed and strongly upregulated by heat and other stresses. We infer from the downregulation of this gene that WGO lowered one or more stressors in larval cells. We note relatively few differences in gene expression between the third instar larvae taken from unsupplemented- and WGO-supplemented media. This accords with classical research on insect essential fatty acid requirements (Dadd, 1985). For many insect species, the eggs provide essential fatty acids to the embryonic and early-post embryonic stages and essential fatty acid deficiency does not appear until the pupal/adult molt. In some species, such as the mosquito *Culex pipiens*, the deficiency does not appear until after the adults emerge and in still other species essential fatty acid deficiency does not appear until the next generation. For the Oriental fruit fly, preliminary results indicate that WGO deficiency does not influence gene expression in newly emerged adults (Chang and Coudron, unpublished observations), but does influence gene expression in 11-day old unmated adults (Chang et al., 2010). We infer from our data on protein spot L-10 that WGO deficiency exerts a subtle stress in fruit fly larvae that increases as the larvae progress through adulthood and egg production.

WGO supplementation led to increased expression of two ubiquitin-associated proteins in F_1 eggs. Expression of the gene for 26S proteasome subunit (spot E-06) increased 5.4-fold. This protein is involved in the ubiquitin–proteasome pathway that degrades most cytosolic and nuclear proteins (Hershko and Ciechanover, 1998). Protein spot E-08, which increased in expression by about 2.5-fold, is a nascent polypeptide-associated complex protein. These proteins reversibly and specifically bind to ribosomes, where they act in protein folding and targeting (Wegrzyn et al., 2006). Expression increased for two genes encoding proteins associated with energy and metabolism, including aconitate hydratase (E-68) and glyceraldehyde-3-phosphate dehydrogenase (E-98). Aconitate hydratase is an iron–sulfur protein that catalyzes isomerization of citrate to isocitrate via cis-aconitate in the TCA cycle (Beinert et al., 1996) and glyceraldehyde-3-phosphate dehydrogenase catalyzes a step in glycolysis (Fife and Szabo, 1973). The expression of a gene encoding Receptor of Activated C Kinase 1 (RACK1; protein spot E-93) was increased by 6.8-fold in F_1 eggs

Table 3. Proteins From Bactrocera dorsalis Egg Homogenates Identified Using Mascot

Spot no.	*Putative protein with accession no. (species)*	*Calculated mass*[a]	*Calculated pI*[a]	*MOWSE protein score*[b]	*Total ion score*[c]	*No. peptides*[d]	*% Protein coverage*[e]	*E-value*[f]	*Peptide sequence (with highest ion score)*[g]
E-06	GJ11829 *Drosophila virilis* gi|195377688	42,362	4.72	230	175	14	39	1.7e–17	IVVFVGSPINNEEGELVK (58)
E-08	GK17972 *Drosophila willistoni* gi|195442105	23,353	4.63	364	312	9	45	6.8e–31	NPHSDTYIVFGEAK (82)
E-17	Glyceraldehyde-3-phosphate dehydrogenase *Glossina morsitans* gi|74950265	35,868	6.96	270	222	11	31	1.7e–21	VPTPNVSVVDLTVR (110)
E-18	GF19402 *Drosophila ananassae* gi|194768002	21,952	5.52	185	169	4	36	5.4e–13	DYGVLDEETGIPFR (115)
E-20	GG21776 *Drosophila erecta* gi|194880679	22,160	8.98	295	262	8	37	5.4e–24	VTDKVFFDITIGGEPAGR (118)
E-24	Similar to *Drosophila melanogaster* CG2852 *Drosophila yakuba* gi|38048603	15,122	8.78	257	217	7	48	3.4e–20	LKHYGAGWLSMANAGK (115)
E-25	Chain A, Crystal Structural Analysis of *Drosophila melanogaster* dUTPase gi|209156721	17,434	5.72	99	74	7	47	0.0002	TDLQVQVPEGSYGR (45)
E-41	Similar to *Drosophila melanogaster* CG4800, *Drosophila yakuba* gi|38048345	16,872	4.75	110	92	8	46	7.9e–05	LQECFAFGDKK (60)
E-45	CG13551, isoform C *Drosophila melanogaster* gi|24659355	9,711	6.29	228	154	9	68	2.7e–17	EHEEAIQR (59)
E-46	Polyubiquitin *Tribolium castaneum* gi|21314339	12,686	5.91	378	294	8	77	2.7e–32	IQDKEGIPPDQQR (107)

Table 3. Continued

Spot no.	*Putative protein with accession no. (species)*	*Calculated mass*[a]	*Calculated pI*[a]	*MOWSE protein score*[b]	*Total ion score*[c]	*No. peptides*[d]	*% Protein coverage*[e]	*E-value*[f]	*Peptide sequence (with highest ion score)*[g]
E-49	GA15351 *Drosophila pseudoobscura pseudoobscura* gi|198457521	89,564	5.20	664	295	53	62	6.8e–61	VTQGFSGADLTEICQR (121)
E-52	GH18558 *Drosophila grimshawi* gi|195036830	35,690	9.21	422	367	13	42	1.1e–36	LFGVSTLDVVR (100)
E-64	14-3-3 epsilon isoform C *Drosophila melanogaster* gi|24647891	29,326	4.74	325	200	20	60	5.4e–27	YLAEFATGSDR (70)
E-68	Aconitate hydratase, putative *Pediculus humanus corporis* gi|212511520	83,002	8.08	148	118	18	25	2.7e–09	IPFNVTPGSEQIR (70)
E-69	Heat shock protein 70 *Culex quinquefasciatus* gi|170049586	38,287	7.56	124	118	6	17	6.8e–07	ALEIDPKNAEALEGYR (108)
E-83	Vitellogenin 1 precursor *Bactrocera dorsalis* gi|19880523	47,922	7.10	403	281	21	57	8.6e–35	VGDVDFYVNGPASAAPGA-TNVIEATMR (118)
E-91	Glyceraldehyde-3-phosphate dehydrogenase (Gadph-2) protein (EC 1.2.1.12) *Drosophila melanogaster* gi|157478	35,532	8.75	240	159	19	61	1.7e–18	LISWYDNEFGYSNR (88)

E-92	GJ20492 *Drosophila virilis* gi|195382366	35,505	8.26	405	342	13	37	5.4e–35	VPTPNVSVVDLTVR (123)
E-93	RACK1 *Drosophila melanogaster* gi|2290597	36,208	7.61	254	129	18	53	6.8e–20	DVLSVAFSADNR (89)
E-97	Heat shock-like protein, similar to heat shock 70 kDa proteins *Ceratitis capitata* gi|662802	71,435	5.36	939	772	36	63	2.2e–88	QTQTFTTYSDNQPGV-LIQVYEGER (155)
E-98	GK17768 *Drosophila willistoni* gi|195457062	35,438	8.26	165	133	11	39	5.4e–11	LISWYDNEFGYSNR (71)
E-102	GK11787 *Drosophila willistoni* gi|195444595	14,523	5.28	95	64	4	17	0.00059	LVQEQKGDKPTTIVR (64)

[a]As reported in Mascot search results.
[b]MOWSE protein scores >75 are significant ($P<0.05$) for the Metazoa search.
[c]Ion scores >44 are significant ($P<0.05$).
[d]The number of peptide sequences identified by Mascot that contributed to the MOWSE Score.
[e]The percent of the protein sequence that is accounted for by the matching peptide sequences.
[f]E-value generated by Mascot.
[g]Sequences of the peptides with the highest ion scores. The individual ion scores are shown in brackets.

Table 4. Relative Changes in Gene Expression and Function of Proteins Identified by MS/MS of Homogenates of Eggs From Adults that were Reared as Larvae on Diet With or Without Wheat Germ Oil (+WGO or −WGO, respectively)

Spot no.	*+WGO/-WGO ratio*	*t-Test P-value*	*Function (category)*
Energy and metabolism			
E-17	0.40	0.003	Plays an important role in glycolysis and gluconeogenesis
E-45	0.23	0.010	Mitochondrial ATPase inhibitor: involved in the negative regulation of nucleotide metabolic processing
E-49	1.57	0.000	AAA+ATPase domain: functions as molecular chaperones, these domains are often subunits in proteases or helicases
E-52	0.43	0.052	Malate dehydrogenases: important in the TCA cycle
E-68	2.43	0.026	Plays important role in TCA cycle
E-83	1.48	0.020	Major yolk protein, serves as a nutrient source for developing embryos
E-91	0.44	0.003	Glyceraldehyde-3-phosphate dehydrogenase: plays an important role in glycolysis and gluconeogenesis
E-92	0.37	0.012	Glyceraldehyde-3-phosphate dehydrogenase: plays an important role in glycolysis and gluconeogenesis
E-98	3.21	0.019	Glyceraldehyde-3-phosphate dehydrogenase: plays an important role in glycolysis and gluconeogenesis
Protein metabolism, structure and function			
E-06	5.43	0.015	26S proteasome subunit: plays a major role in protein breakdown.
E-08	2.48	0.039	Protein domains[a]: (1) Nascent polypeptide-associated complex protein, reversibly binds ribosomes; (2) ubiquitin-associated domain
E-20	0.01	0.016	Cyclophilin: involved in protein folding, can modulate protein function
E-24	0.09	0.035	Cyclophilin: involved in protein folding, can modulate protein function
E-46	0.12	0.021	Involved in the regulated turnover of proteins important in cell cycle progression
Protein structure and signal transduction			
E-69	1.85	0.006	Involved in protein folding and signal transduction
E-97	0.49	0.044	Involved in protein folding and signal transduction
Intracellular signaling			
E-64	1.88	0.035	Numerous impacts on intracellular signaling, including: direct regulation of bound protein; sequestration or modification of molecules; subcellular localization of bound ligand
E-93	6.80	0.015	Anchors activated protein kinase C's to subcellular compartments
Lipid transport			
E-102	0.36	0.038	Lipocalin family: transports small hydrophobic molecules, such as lipids, steroid hormones, and retinoids; includes prostaglandin D synthase
Cell protection			
E-18	0.11	0.000	Thioredoxin peroxidase: reduces hydrogen peroxide, peroxynitrite, and organic hydroperoxides
DNA replication and repair			
E-25	0.41	0.013	DUTPase: hydrolyses dUTP to dUMP and pyrophosphate
E-41	0.26	0.000	Exact function unknown; able to bind to tubulin in the cytoskeleton, has a high affinity for calcium, and is induced in vitamin D-dependent apoptosis

[a]Protein domains identified by MotifScan, http://myhits.isb-sib.ch/cgi-bin/motif_scan.

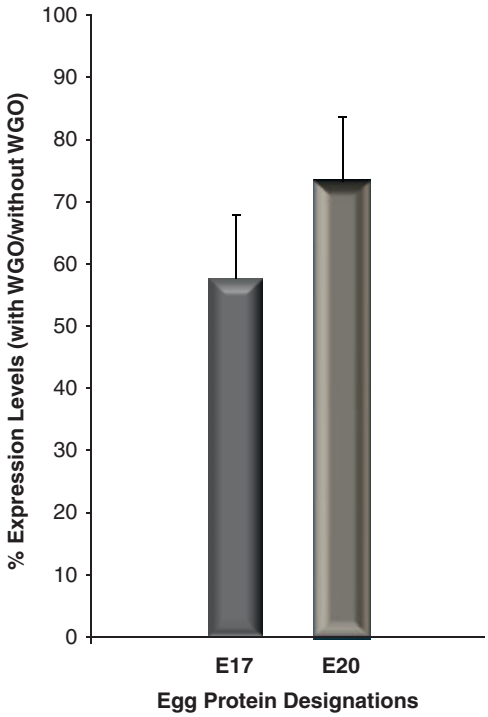

Figure 3. WGO influence on mRNA levels in eggs oviposited by adults from larvae reared on diet with and without WGO. Gene-specific primers were generated based on sequence data from selected proteins and were used to determine mRNA levels using quantitative PCR. Histogram bars represent mean+SE. WGO, wheat germ oil.

from fruit flies reared on WGO-supplemented medium. RACK1 acts in various intracellular signal transduction pathways involving protein kinase C. Because protein kinase C acts in several signaling pathways, RACK1 is an important component of intracellular signaling. RACK1 is known from many eucaryotic cells, including insect cells. In the spruce budworm, *Choristoneura fumiferana*, for example, RACK1 and protein kinase C are necessary for the 20-hydroxyecdysone-stimulated expression of a molt-associated transcription factor (Quan et al., 2006). We speculate that one of the subtle effects of essential lipid deficiency, as seen in eggs from fruit flies reared on WGO-lacking medium, is an unseen limitation in signal transduction potential.

Proteins that decreased in expression in F_1 eggs by twofold or more as a result of supplementing the larval diet with WGO included two forms of cyclophilin (E-20 and E-24) and thioredoxin (E-18), which facilitate protein folding and isomerization of peptide bonds (Schonbrunner et al., 1991; Berndt et al., 2008), and polyubiquitin (E-46). These proteins are involved with regulating the translocation and degradation of cellular proteins (Li and Ye, 2008). We also recorded a decrease in proteins associated with stress or immune functions, including a HSP70-like protein (E-53) and lipocalin (E-102), a transport protein involved with immune response (Flower, 1996), dUTPase (E-25), thought to prevent misincorporation of dUMP into DNA during replication and repair (Chen et al., 2002), and CG4800 (E-41) named a translationally controlled tumor protein but its function is poorly understood (Guillaume et al., 2001). The expression level of five proteins associated with energy and metabolism decreased by twofold or more including three proteins associated with glyceraldehyde-3-phosphate

dehydrogenase (E-17, E-91, and E-92), best known for its role in glycolysis, a mitochondrial ATPase inhibitor (E-45) involved in decreasing cellular and mitochondrial ATP levels (van Raaij et al., 1996), and malate dehydrogenase (E-52), involved in the citric acid cycle and gluconeogenesis (Minarik et al., 2002). The downregulation of glyceraldehyde-3-phosphate dehydrogenase(s) and malate dehydrogenase suggest that the addition of WGO to the diet leads to reduced carbohydrate metabolism in the fruit fly.

Turning to the protein identification information (Tables 3 and 4), we note high significance levels by MOWSE scores and E-values. We regard this as indication of homology in the part of the sequence related to that functionality of the protein.

Organisms compensate for alterations in nutritional quality to maintain critical homeostasis appropriate to given stages of development and to minimize the impact of the nutritional shortcomings. In our view, the ideal biological markers are associated within the compensation processes (e.g. energy production and cell protection), rather than a major activity associated with a specific developmental stage (e.g. vitellogenesis; Coudron et al., 2006). Hence, we propose that genes in the egg demonstrated pronounced expression level changes, i.e. E-06, E-20, E-24, are appropriate biomarker candidates for additional study.

As we saw for protein L-10, a few other proteins recorded from eggs and larvae were also expressed in adults (Chang et al., 2010). For larvae, L-26, another heat shock protein, corresponds to protein F-40 and F-114, which did not significantly increase in expression in females. For eggs, E-17 agrees with M-53; expression of this protein decreased by about 60% in eggs and males. E-49 equates to F-06; although expression in eggs did not increase, F-06 increased by about 4-fold in adults reared on diets supplemented with WGO. Egg protein E-52 matches female protein F-46; expression did not change significantly for either developmental stage. E-92 is the same as F-54, expression of which is significantly decreased in eggs and adult females. L-13 matches M-03, which in males reared on WGO-supplemented diets increased by twofold while expression of L-13 decreased by about 40%. Proteins that undergo such excursions in expression over development may be important in identifying biomarkers for nutritional deficiency.

Early stages in larval development may be the preferred stage for discovery of molecular markers for nutritionally deficient culture media because media development decisions could be taken with minimal rearing time and costs. As mentioned earlier, this may be biologically inappropriate if important nutrients are provided in the eggs. Nonetheless, for optimal media development, the F_1 egg may be a preferred stage. Egg stages also provide purity of sample, and ease of collection, storage and handling. This has the down-side of rearing insects through to F_1 eggs before a decision on media quality can be taken. These findings apply to the Oriental fruit fly; with respect to the broader issue of efficiently developing insect culture media, we emphasize the importance of continued research on the use of biomarkers for media development and with other insect species.

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