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Identification of protein related to dietary vitamin B_3 deficiency in Mediterranean fruit fly larvae

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Abstract

Mediterranean fruit fly (medfly), *Ceratitis capitata*, is among the most destructive agricultural pest. The sterile insect technique (SIT) can effectively control medfly populations. To rear healthy medflies for the purpose of SIT, it is essential to supplement B vitamins in the diet. However, the function of the dietary B vitamins in *C. capitata* larvae is not known. With the microscopic analysis, several organs in the head were examined and the spiracle formation and sensory organs were normally formed between the niacin-supplied and niacin-absent groups. However, formation of the ocular depression was differently developed between the two groups, although the hypostomal sclerite was formed properly. These results signify that niacin deficiency maybe interrupt development of medfly larvae ocular depression. Proteomic analyses using LC MS/MS detected a total of 1845 proteins in two flies. A total of 607 of the 1845 proteins were overexpressed and one third (598 proteins) were downexpressed in the niacin-deficient larvae, while about one third were similarly expressed. Overexpressed proteins in the niacin-deficient larvae included ryanodine receptor 44 F, intergrin-PS, spalt-major homeotic protein, and chiffon protein. One of important overexpressed proteins was optomotor-blind protein in relation to wing development in the niacin-deficient medfly larvae.

KEYWORDS
insect, nutrition: eye, tephritid fruit fly, vitamin B3, vitamin deficiency

1 | INTRODUCTION

Tephritid fruit flies are economically significant pests worldwide. Its control mainly relies on sterile insect technique (SIT). SIT requires continuous mass rearing, sterilization of adult flies, and release of sterilized male flies into fields to compete with wild male flies for female flies. A female that mates with a sterile male will not produce offspring. Repeated release of sterile males can effectively suppress the population. The Mediterranean fruit fly (medfly), *Ceratitis capitata*, is not only one of the most destructive agricultural pests but also one of the most successful SIT controls in tephritids.[1] Dietary B vitamins affect medfly growth, but the mechanism through which these effects occur is not known. Medfly larvae require B vitamins for normal development.[2,3] Vitamin B3, which is also known as nicotinic acid or niacin, plays an important role in larval development and is a component of nicotinamide adenine dinucleotide (NAD).[4] Medfly larvae reared on a niacin-free diet fortified with 707 μg/g of nine other vitamins were found to have body sizes approximately one third that of healthy larvae (Figure 2II) and suffered 100% mortality in the second instar.[5] These mortality rates are inversely correlated with the concentrations of nicotinamide in both larvae and diet.[6] These findings indicate biochemical evidence that nicotinic acid (involved

in NAD biosynthesis) is essential in *C. capitata* larval development and growth.[6] The *C. capitata* may be a good animal model to study and find whole side-effects of nicotinamide and niacin deficiencies on behalf of mammals. Functional analyses using omics techniques can provide clues relating to side-effects of the deficiency affecting NAD homeostasis in the insect. Therefore, proteomic and genomic studies were conducted to find the proteins and genes differentially expressed in the niacin-deficient larvae of the insect compared to the control. The differentially expressed proteins and genes were cross-checked using biochemical and molecular biological experiments.

The objectives of this study were to compare protein profiles in the niacin-deficient larvae and healthy larvae, to explore the possible cause of mortality of niacin in the larvae and understand possible molecular mechanisms of the effects, protein network, and responses to niacin in the larvae of the medfly. To our knowledge, this is the first comprehensive profile of soluble proteins in the niacin-deficient larvae of the medfly which were reared on a niacin-free diet fortified with 707 μg/g of nine other vitamins had a body size approximately one third that of healthy larvae and suffered 100% mortality in the second instar.

2 | MATERIALS AND METHODS

2.1 | Laval sample preparation

Ceratitis capitata larvae were reared on *C. capitata* No. 1 diet.[3] The larvae were raised according to the procedure described in a previous study.[6] The healthy larvae and niacin-deficient larvae were collected from groups provided each test diet and rinsed thoroughly with distilled water for 6 days after egg seeding. Two grams of larvae were then incubated in 10 mL of lysis buffer [40 mM Tris-HCl, pH 7.4, 5 mM dithiotreitol [DTT], 1 mM phenylmethylsufonyl fluoride (PMSF)] and 500 μL of protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland) at room temperature for 15 min. The lysates were then homogenized on ice using an Ultraturrax homogenizer (Cole Parmer, Verman Hills, IL) for 3 min at an interval of 30 s, after which the homogenate was centrifuged at 17 555 *g* for 30 min. The supernatant fractions were saved for protein identification by nano LC/IT/MS, while the protein concentration was determined using a Coomassie PlusTM Protein Assay Kit (Pierce). Equal amounts (25 μg) of protein samples were then subjected to electrophoresis through 4-20% polyacrylamide SDS-containing gels.

2.2 | One-dimensional (1D) SDS-PAGE, protein digestion, and peptide extraction

An aliquot of protein extracts from larvae (1.5 μL) (25 μg protein equivalents) was mixed with SDS-PAGE sample buffer (3.0 μL) and heated at 100°C for 5 min. The denatured proteins were separated on 10-20% gradient SDS-PAGE mini gels (9 × 10 cm, PAGE Gold Precast Gel, Cambrex Bioscience, Rockland, ME) followed by Coomassie dye G-250 staining. Each gel lane was cut into 20 even slices, destained with 50% v/v acetonitrile (ACN) in 25 mM NH_4HCO_3, and then completely dried in a speed-vacuum centrifuge (Eppendorf, Hamburg, Germany) after dehydration with ACN. The dried gel slices were reduced in 50 μL of 10 mM DTT for 45 min at 56°C, alkylated in 50 μL of 55 mM iodoacetamide for 45 min at ambient temperature in the dark. The gel slices were dehydrated with ACN followed by drying in the speed-vacuum centrifuge. After addition of 20 μL of sequencing-grade modified porcine trypsin (20 ng/μL in 50 mM NH_4HCO_3), samples were incubated at 37°C overnight. Tryptic digestion was stopped by adding 5 μL of 2% trifluoroacetic acid (TFA). The digested peptides were extracted from each gel slice by sonication with 30 μL of ACN/H_2O/TFA (50:49.9:0.1, v/v/v) twice by sonication for 45 min on ice.

2.3 | Two-dimensioned gel electrophoresis (2D GE) for identification of phosphorylated proteins

Two-dimensioned gel electrophoresis (2D GE) was conducted using a Protein IEF Cell (BioRad) with 110 mm pH 3-10 IPG strips and Criterion Precast Gels. A total of 250 mg of wet larval samples were added to 10 mL of protein extraction buffer (1% Triton X-100, 40 mM Tris-HCl, pH 8.0, 65 mM DTT, 1 mM PMSF, 10 mM aprotinin, and protease inhibitor cocktail) and homogenized using an Ultraturrax homogenizer (Cole Parmer, Vermon Hills, IL) with an Omnitip disposable generator probe (Model CTH-115) at an interval of 30 s on ice (two times). The homogenized samples were centrifuged at 15 292 *g* (4°C) for 30 min, after which 100 μL of the supernatant of each sample (50 μg protein) was prepared for 2D using a GE ReadyPrepTM 2D Cleanup Kit (Bio-Rad, Richmond, CA) according to the manufacturer's protocols. Individual pellets from each cleanup step were then resuspended in 50 μL of 2D rehydration/sample buffer and combined to give a final sample with a total volume of 100 μL. A sum of 2 μL of phosphatase inhibitor cocktail 2 was added to each 100 μL of sample. After that, 2D electrophoresis was performed according to the Protein IEF cell instruction manual (BioRad Catalog No. 165-4000).

2.4 | Microscopic analysis of spiracles and optical oppressions

All microscopic analysis of spiracles and optical depressions was carried out using Leica Microsystems (Wetzlar GmbH, Wetzler, Germany).[7] Images were taken at 20 × 10.50 using a zoom digital camera (Kodak DC290, Tokyo, Japan).

Fifty fresh larvae were transferred to RNeasy lysis buffer that was supplied with the RNeasy® mini kit and the total RNA from larval samples was extracted using an RNeasy® mini kit (Qiagen Inc., Valencia, CA) and then measured with a Beckman DU® 530 Life Science UV/VIS spectrophotometer in accordance with the manufacturer's protocols. Sufficient amounts of RNA with adequate purity for subsequent analyses were obtained.

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2.5 | Microarray data analyses

GeneChip hybridizations were carried out at the Greenwood Molecular Biology Core Facility, University of Hawaii. Briefly, 15 μg of total RNA was processed on each Drosophila GeneChip (Affymetrix Inc., Santa Clara, CA) for microarray measurements. RNA amplification was then conducted according to the Affmetrix GeneChip® expression analysis technical manual using the GeneChip® one-cycle. The yields of cRNA for each sample were adjusted to 61.0 μg in the control sample and 46.0 μg in the niacin-deficient larvae. The fragment cRNA was posthybridized to the Affymetrix GeneChip Drosophila Genome Array, which contains 8000 transcriptions of 13 500 genes, after which the arrays were scanned with an Affymetrix Gene Array® 2500 scanner using Affymetrix Microarray Suite 5.0 (MAS) and the resulting microarray images were quantified based on the fluorescence data from the images using MSA 5.0. Following background correction and normalization, transcription abundance was estimated using fluorescence signal intensities. Significance analysis of microarrays[8] and supervised learning software for genomic expression data mining was applied to identify differentially expressed genes.

2.6 | Protein identification by LC-ion trap mass spectrometry (LC-ITMS)

Analyses of the digested peptides were performed using a Dionex UltiMateTM 3000 nano LC interfaced with a Bruker esquireHCT ultra ion trap mass spectrometer in nanoelectrospray mode with a PicoTip Emitter (360 μm O.D., 20 μm I.D., 10 μm tip I.D., New Objective, Woburn, MA, USA) according to the procedure previously published.[9,10] The nano-LC column was C18 PepMap 100 (3 μm film thickness, 75 μm I.D. × 15 cm, Dionex Corp., Sunnyvale, CA) at a flow of ca. 180 nL/min. MS/MS spectra were interpreted with Mascot (Matrix Science, London, UK) via Biotools 2.2 software (Bruker). Peak lists for protein identification were created by Compass 1.3 of Bruker for esquireHCTultra. Peptide mass fingerprint (PMF) searches and sequence alignments were performed in Swiss-Prot through the Mascot sever with database of *Drosophila melanogaster*. UniProt classification was used to search cellular functions of identified proteins. Peptides were assumed to be monoisotopic, oxidized at methionine residues, and carbamidomethylated at cysteine residues. Up to one missed trypsin cleavage was allowed, although matches that contained any missed cleavages were not noticed. Peptide mass and MS/MS tolerances were set at ±1.0 and ±0.8 Da, respectively. Probability-based molecular weight search (MOWSE) scores were estimated and were reported as: 10 x log10 (*P*), where *P* is the absolute probability. Scores in Mascot larger than the MOWSE score at $P = .05$ were considered statistically significant, meaning that the probability of the match being a random event is lower than 0.05. The false-positive rate (FPR) was estimated according to the method of Elias et al.[11] and was smaller than 1% [FPR = FP/(FP + TP), where FP is the number of FPR hits; TP is the number of true-positive hits]. Only proteins identified with at least two peptide hits, with each peptide containing two tryptic termini, were accepted. In addition, the MS/MS spectra of all positively identified peptides were manually confirmed twice. Proteins of the healthy medfly larvae and niacin-deficient medfly larvae were identified in triplicate.

2.7 | Quantitative real-time PCR of org-I

Quantitative real-time PCR (qPCP) experiments were performed using an Applied Biosystems Step OneTM PCR system (Foster, CA). The primer pairs used in the reaction were 5′-GACAACAACAGT GCGGAGAA-3′ (forward) and 5′-TGAAAGTGCGAAAGTGGC-3′ (reverse), 5′-GGACACCAGCCTAACGGACTA-3′ (forward) and 5′-TGAT GTGCAGCCGAACC-3′ (reverse), 5′-CGAGCATTGGACAACCCTAT-3′ (forward) and 5′-TGGTAGCCCAGCAAGTCG-3′ (reverse). Two-step qPCR was performed in two separate reactions. First, total RNA was reverse transcribed into cDNA, after which the cDNA was amplified by PCR (High Capacity cDNA reverse transcription kit, Part No. 4368814, Applied Biosystems). cDNA synthesis was carried out using a Takara PCR Thermal Cycler Dice (Takara Bio) at room temperature by heating the samples at 37°C for 30 min and then 42°C for 30 min, and then cooling them to 4°C (cycle I). For comparison, samples were subjected to 95°C for 5 min followed by 30 cycles of 95°C for 30 s, 65°C for 30 s, and 72°C for 1 min (cycle II) and then final extension at 72°C for 10 min. Each qPCR reaction was run three times.

3 | RESULTS AND DISCUSSION

3.1 | Differential expression of proteins in healthy larvae and niacin-deficient larvae

LC-MS/MS revealed a total of 1845 proteins, among which 1237 and 1243 proteins were identified in larvae reared on a diet with 10 B vitamins (healthy larvae) and in those reared on a niacin-free diet containing 9 B vitamins (niacin-deficient larvae), respectively (Supporting information Tables S1 & S2 and Figure 2II). The 605 and 519 proteins were up-/downregulated in response to deficiency of niacin, respectively (Supporting information Tables S3 & S4 and Figure 2II). Niacin deficiency affects the calcium-mediated, sevenless, mitogen-activated protein kinase (MAPK), smoothened and wingless signaling pathways (Table 1). In the present study, calcium-mediated signaling pathways in the niacin-deficient larvae were upregulated via over-representation of G-protein coupled receptor kinase 1, a photoreceptor specific protein kinase C (eye-PKC), and diacylglycerol (DAG) kinase that attenuates PKC activity (Table 1). The downstream target proteins of the calcium signaling pathway are ryanodine receptor 44 F, integrin-PS, spalt-major homeotic protein, and chiffon protein[12–14] (Table 1). Ryanodine receptor 44F, which is referred to as the intracellular calcium channel, is probably essential for excitation-contraction coupling in the larval body wall muscle.[15] Integrin-PS works as a receptor for dorsal closure and somatic muscle attachments and organization of ommatidial arrays of the larval eye imaginal disk.[16] The spalt-major homeotic protein encoded by spalt-related participates in sensory

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TABLE 1 Major over-represented proteins of medfly (*Ceratitis capitata*) in response to deficiency of niacin

Protein name (Gene symbol for IPA using human benign database)	No. of matched peptides	Mascot Score ($P = .05$)	Accession number	Biological function
A. PKC activation or calcium-mediated signaling pathway				
G-protein coupled receptor kinase 1 (Gprk1) (*ADRBK2*)	8	42 (30)	P32865	Imaginal disc-derived wing vein specification
Inositol 1,4,5-triphosphate receptor (InsP3R) (*ITPR1*)	11	74 (30)	P29993	Visual and olfactory transduction
Protein kinase C, eye isozyme	4	34 (27)	P13677	Photoreceptor-specific PKC (Eye-PKC)
Protein kinase C, brain isozyme (*PRKCA*)	5	31 (28)	P05130	Intracellular signaling cascade
Diacylglycerol kinase (*DGKB*)	8	45 (37)	Q01583	Initiating the resynthesis of phosphatidylinositols and attenuating protein kinase C activity
Putative protein kinase C, delta type	4	34 (28)	P83099	MAP kinase signaling
Calcium-binding mitochondrial carrier aralar 1 (*SLC25A12*)	7	40 (28)	Q9VA73	Calcium-dependent mitochondrial solute carrier
Ryanodine receptor 44F	14	40 (37)	Q24498	Calcium transport
Integrin -PS	5	46 (31)	P11584	Organization of ommatidial arrays (larval eye and wing imaginal disk)
Homeotic protein spalt-major (*SALL3*)	4	29 (26)	P39770	Photoreceptor
Chiffon protein	13	84 (31)	Q9NK54	A role in eye and thoracic bristle development
B. Sevenless signaling pathway				
Disabled protein	7	48 (27)	P98081	Sevenless signaling
Protein enhancer of sevenless 2B (*GRB2*)	4	32 (31)	Q08012	Ras protein signal transduction and activation of JNKK
T-related protein (Trp) (*T*)	15	39 (37)	P55965	Hindgut and anal pad specification (Brachyenteron protein)
Transient receptor potential protein	7	41 (37)	P19334	Light-sensitive channels
C. MAPK signaling pathway or related proteins				
Mitogen-activated protein kinase kinase kinase (mixed lineage kinase) MAPKKK	6	33 (28)	Q95UN8	Activates the JUN N-terminal pathway during dorsal closure
MAP kinase-activating death domain protein(*MADD*)	26	83 (32)	Q9VXY2	Apoptosis
Ras GTPase-activating protein (*RASA3*)	12	86 (30)	P48423	Ras protein signal transduction
Epidermal growth factor receptor precursor (EGFR)	9	78 (30)	P04412	Signal transduction through the ras-raf-MAPK pathway
Optomotor-blind protein (*TBX2*)	9	40 (29)	Q24432	Transcription regulator/compound eye morphogenesis
Raf homolog serine/threonine protein kinase dRAF-1 (*BRAF*)	4	43 (32)	P11346	MAP kinase signaling
Heat shock protein 83 (Hsp83) (*HSP90AB1*)	10	75 (31)	P02828	RAF-mediating signaling molecular chaperone.
Heat shock protein 70 (Hsp70)	8	66 (31)	Q9GSU4	Heat shock induces the synthesis of seven proteins
Stress-activated protein kinase JNK	4	29 (26)	Q966Y3	Stress, signal transduction
Tyrosine-protein kinase (*TEC*)	9	64 (30)	P08630	Required for proper ovarian ring canal development
PHD finger protein rhinoceros	28	141 (31)	Q7YZHI	Negative regulator of the EGFR/Ras/MAPK signaling pathway during eye development
14-3-3 protein zeta (*YWHAZ*)	5	249 (28)	P29310	Raf-dependent cell proliferation and photoreceptor differentiation during eye development

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TABLE 1 (Continued)

Protein name (Gene symbol for IPA using human benign database)	No. of matched peptides	Mascot Score ($P = .05$)	Accession number	Biological function
D. JAK/STAT signaling pathway				
Tyrosine-protein kinase hopscotch (JAK)	5	50 (28)	Q24592	Control of pair-rule gene transcription in a stripe-specific manner
E. Smoothened signaling pathway				
Smoothened protein precursor (dSMO) (*SMO*)	9	45 (30)	P91682	Segment polarity protein
Protein shifted precursor (WIF-1-like protein)(*WIF1*)	3	33 (31)	Q9W3W5	Smoothened signaling pathway (WNT inhibitory factor 1)
F. Wg/Wnt signaling pathway				
Protein kinase shaggy (*GSK3B*)	5	30 (27)	P18431	Synctial blastoderm formation and embryonic segmentation/meiosis
Wingless receptor (dfz2)	4	40 (27)	S71786	Wnt signaling pathway
Frizzled-2 (*FZD5*)	8	62 (31)	Q9VVX3	Wnt signaling pathway (receptor for Wnt proteins)
Glucosamyl *N*-deacetylase/*N*-sulfotransferase (*NDST2*)	8	36 (31)	Q9V3L1	Wnt signaling pathway
Protein pangolin (*TCF7L2*)	3	42 (27)	P91943	Wingless (Wg) signal in embryos and in developing adult tissues/transcription factor 7-like 2 (T-cell specific, HMG-box)
Exostosin-1 (*EXT1*)	7	44 (31)	Q9V730	Wnt signaling pathway
Exostosin-3 (*EXTL3*)	8	32 (31)	Q9XZ08	Wnt signaling pathway/Exostoses (multiple)-like 3
Protein groucho (*TLE3*)	6	42 (30)	P16371	Negative regulation of Wnt receptor signaling pathway/transducin-like enhancer of split 3
Apolipophorins	16	99 (30)	Q9V496	Lipid transport/Wnt signaling pathway (retinoid-and fatty acid-binding glycoprotein)
G. CNS development or proteins present in neurons				
Locomotion-related protein Hikaru genki precursor	7	44 (31)	Q09101	Development of CNS; functional neural circuits
α-adaptin homolog (*AP2A2*)	9	58 (31)	P91926	Presynaptic vesicle recycling/adaptor-related protein complex 2, α 2 subunit
Serrate protein (*JAG2*)	7	54 (31)	P18168	Notch receptor essential ectodermal development/Jagged 2
Still life protein	14	98 (31)	P91621	Regulate synaptic differentiation through the organization of the actin cytoskeleton by activating Rho-like GTPases
Rab3 GTPase-activating protein catalytic subunit 1 (*RAB3GAP1*)	9	59 (31)	Q9VQ26	Regulation of GTPase activity
Netrin-B (*NTN1*)	7	49 (31)	Q24568	Netrins control guidance of CNS commissural axons and peripheral motor axons/Netrin 1
Ubiquitin ligase protein highwire (*MYCBP2*)	23	89 (31)	Q9NB71	Ubiquitin ligase conjugation pathway and adult walking behavior and locomotion/MYC binding protein 2
H. Metamorphosis				
Krueppel homologous protein	10	45 (31)	P08155	Metamorphosis, Ecdysone
RNA-binding protein cabeza (*FUS*)	7	66 (31)	Q27294	Adult locomotory behavior/fused in sarcoma
Nuclear hormone receptor HR38	8	39 (28)	P49869	Binds to NGFI-B response elements/late stages of epidermal metamorphosis

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TABLE 1 (Continued)

Protein name (Gene symbol for IPA using human benign database)	No. of matched peptides	Mascot Score ($P = .05$)	Accession number	Biological function
Protein lap4 (*SCRIB*)	11	73 (31)	Q7KRY7	Polarization of the embryonic, imaginal disk and follicular epithelia/Neurogenesis, Olfaction, Sensory transduction/Scribbled homolog (*Drosophila*)
I. Others				
Hook protein (*HOOK3*)	9	34 (28)	Q24185	Stabilizing organelles of the endocytic pathway
Apoptosis 2 inhibitor (*BIRC3*)	3	30 (27)	Q24307	Apoptosis (Apoptotic suppressor, Over-expression suppresses RPR and HID-dependent cell death in the eye)
Tyrosine-protein kinase PR2	13	67 (30)	Q9I7F7	Salivary gland cell autophagic effect of the gypsy transposable element
Suppressor of hairy wing protein	9	68 (31)	Q08876	Phenotypic effect of the gypsy transposable element.
Transcription factor glial cells missing	9	50 (31)	Q27403	Induces gliogenesis
Period circadian protein (Protein clock-6)	4	40 (32)	P07663	Biological clock functions/Biological rhythms/age-dependent response to oxidative stress
Fructose-bisphosphate aldolase (*ALDOA*)	11	296 (30)	P07764	Glycolysis
*Protein sex-lethal (*sxl*)	4	33 (30)	P19339	Sexual differentiation

†Results are LC-MS/MS data processed with MASCOT search engine with a database of Drosophila melanogaster and the sequence alignments. UniProt and TIGR classification were used to search cellular roles of identified proteins. JNK was only matched with Suberites domuncula.
‡Evolution of sex determining cascades [Pane, et al. Development, 129, 3715–3725 (2002)]. Ceratitis and Drosophila sex-determining cascade differ at the level of transformer as well as upstream of it. The gene has conserved its function during evolution, but it has female-specific positive auto regulation in Ceratitis, while in Drosophila it needs Sxl as upstream regulator to express its female determining function. As Ceratitis capitata is a major agricultural pest in many areas of the world, the isolation of a key sex-determining gene such as Cctra will substantially aid the development of new strategies to optimize the efficacy of currently used male sterile techniques for pest control.

organ development or photoreceptor cell differentiation including R8 cell differentiation.[17] Chiffon protein has the additional phenotypes of rough eyes and thin thoracic bristles.[18] Overexpressed proteins involved in the sevenless signaling pathways in niacin-deficient larvae include disabled protein, protein enhancer of sevenless 2B, T-related protein (Trp), and transient receptor potential protein (Table 1B). Trp plays a role in hindgut and anal pad specification[19] and serves as a light-sensitive phototransduction channel.[20] *Opotomotor-blind* (*omb*) protein is a transcription factor that regulates key processes involved in eye development (Table 1) and is particularly involved in counteraction of eye formation and loss of the arista.[21] Zinc finger protein 2, kinesin-like protein costa, protein apterous, carboxypeptidase D, protein dachsous, SP460, and short stop (isoform H) were underrepresented in the niacin-deficient larvae (Table 2). These proteins are related to imaginal disc development via imaginal disc-derived wing/leg morphogenesis. The under-represented protein tumorous imaginal discs and cAMP-dependent protein kinase catalytic subunit are associated with tumor suppression in larval imaginal disks and negative regulation of the smoothened signaling pathway, respectively. The proteins associated with immune response and innate immunity that were downregulated in the niacin-deficient larvae relative to the healthy larvae included protein sickie, Gram-negative bacteria-binding protein 3, and phagocyte signaling-impaired protein (Table 1). Figure 2 shows that photograph of Medfly larvae reared on a niacin-free diet fortified with 707 μg/g of nine other vitamins (I, B), number of protein identified (II), and number of proteins mapped to human ortholog through the Ingenuity Pathway Analysis (IPA) by Ingenuity Knowledge Base (Human/Mouse/Rat data) with protein ID for Pathway and network analysis (III). Venn diagrams (II) in Figure 2 indicates that numbers of proteins differentially identified in the parentheses, of which 640, 598, and 607 proteins were overlapped, underexpressed, and over-expressed, respectively (Figure 2). These results indicate that the eye compound is normally not formed due to inhibition of imaginal disc development by niacin deficiency.

3.2 | Relations between over-expression protein kinase C and ocular depression

We speculate that, when induced by niacin-deficiency, the overexpressed PKC (eye-PKC) plays a major role in prohibiting normal eye development in larvae. Larval sensory organs, spiracles, and eyes of the second-instar larvae were observed under an electron microscope. The spiracles looked very similar between the two groups Figure (1A), showing second-instar characteristics.[22] However, development of the ocular depression between the two group larvae differed dramatically

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TABLE 2 Major over-represented proteins of medfly (*Ceratitis capitata*) in response to deficiency of niacin

Protein name(Gene symbol for IPA using human benign database)	No. of matched peptides	Mascot Score ($P = .05$)	Accession number	Biological function
A. Imaginal disc development				
Zinc finger protein 2	6	58 (31)	P28167	Imaginal disc-derived wing morphogenesis
Kinesin-like protein costa	4	54 (32)	O16844	Imaginal disc-derived wing morphogenesis
Protein apterous (*LHX2*)	4	32 (30)	P29673	Imaginal disc-derived wing morphogenesis
Carboxypeptidase D (*CPD*)	5	34 (31)	P42787	Imaginal disc-derived wing morphogenesis
Protein dachsous (*DCHS1*)	9	53 (32)	Q24292	Imaginal disc-derived leg morphogenesis
SP460	7	48 (37)	Q9U3W7	Ventral imaginal disc-derived wing surfaces
Short stop, isoform H	6	39 (36)	A1Z9J3	Ventral imaginal disc-derived wing surfaces
Chitinase-like protein Idgf5	3	30 (28)	Q8TOR7	Chitin catabolic process
Chitinase-like protein Idgf4	4	36 (32)	Q9W303	Chitin catabolic process
Protein snail	2	39 (32)	P08044	Dorsal/ventral axis specification
Protein winged eye	5	58 (32)	Q3LHL9	Imaginal disc morphogenesis
Protein tumorous imaginal discs (*DNAJA3*)	3	46 (32)	Q27237	Tumor suppressor in larval imaginal disks
Putative fat-like cadherin-related tumor suppressor homolog (*FAT1*)	10	94 (32)	Q9VW71	Foregut morphogenesis (negative regulation of imaginal disc growth)
cAMP-dependent protein kinase catalytic subunit (*PRKACB*)	3	43 (32)	P12370	Anterior/posterior pattern formation, compound eye development, locomotor rhythm
B. Sensory transduction				
Gr32a	6	41 (27)	B5D10	Sensory perception of taste
Kakapo	6	39 (36)	O96936	Sensory organ development
Putative odorant receptor 83c	2	33 (31)	Q9VNK9	Olfaction/sensory transduction
Protein no-on-transient A	7	46 (30)	Q9GRX4	Sensory transduction/vision/mRNA binding
Odorant receptor 59a	3	40 (32)	P81923	Olfaction/sensory transduction
Putative odorant receptor 9a	3	33 (32)	Q9W2U9	Olfaction/sensory transduction
C. Cell proliferation & photoreceptor				
Decapentaplegic protein (*BMP2*)	4	39 (28)	P07713	Negative regulation of cell proliferation
Tyrosine-protein phosphatase Lar (*PTPRD*)	6	49 (30)	P16621	Cell adhesion/R7 cell development /photoreceptor cell morphogenesis
Protein sevenless (*ROS1*)	7	84 (32)	P13368	R7 cell development, R8 cell fate specification, positive regulation of photoreceptor cell differentiation
26S proteasome non-ATPase regulatory subunit 7 (*PSMD7*)	3	35 (32)	P26270	Cell proliferation/proteasome
D. G-protein coupled receptor protein signaling pathway				
Putative gustatory receptor 22c	2	31 (30)	P58952	G-protein coupled receptor
Gr59d	4	30 (27)	Q291Y8	G-protein coupled receptor
Putative gustatory receptor 77a	3	39 (32)	Q8IPU5	G-protein coupled receptor
G-protein coupled receptor moody (*MTNR1A*)	3	38 (32)	Q9W534	G-protein coupled receptor
Putative gustatory receptor 59d	3	39 (32)	P58985	G-protein coupled receptor/sensory perception of taste
G-protein coupled receptor Mth-like 14	2	35 (32)	Q8SYV9	G-protein coupled receptor
Putative gustatory receptor 58c	2	39 (32)	Q9W2B2	G-protein coupled receptor
Probable G-protein coupled receptor	3	32 (31)	P83118	G-protein coupled receptor
Putative gustatory receptor 89a	4	42 (30)	Q9VEU0	G-protein coupled receptor

(Continues)

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TABLE 2 (Continued)

Protein name(Gene symbol for IPA using human benign database)	No. of matched peptides	Mascot Score ($P = .05$)	Accession number	Biological function
Putative gustatory receptor 61a	4	38 (31)	Q9W0M2	G-protein coupled receptor/Sensory perception of taste
Muscarinic acetylcholine receptor DM1 (*CHRM1*)	8	35 (31)	P16395	G-protein coupled receptor
Octopamine receptor beta-1R (*HTR4*)	3	33 (31)	Q9VCZ3	Activation of adenylate cyclase activity
E. Torso signaling pathway				
Tyrosine-protein kinase receptor torso	7	50 (32)	P18475	Torso signaling pathway
Torso-like protein	3	33 (32)	P40689	Terminal region determination/Torso signaling pathway
F. Immune response				
Gram-negative bacteria-binding protein 3	2	38 (32)	Q9NHA8	Immune response/innate immunity
Evolutionarily conserved signaling intermediate in Toll pathway (*ECSIT*)	3	33 (32)	Q9U6M0	Immune response/innate immunity
Protein sickie	7	93 (32)	Q9VIQ9	Immune response/innate immunity
Peptidoglycan-recognition protein SC2 (*PGLYRP4*)	3	34 (32)	Q9V4 × 2	Immune response/innate immunity
Phagocyte signaling-impaired protein (*NAA25*)	2	36 (32)	Q9VDQ7	Immune response/innate immunity
Nuclear pore complex protein Nup88 (*NUP88*)	4	35 (31)	Q9GYU8	Immune response/mRNA transport
G. Protein biosynthesis				
Eukaryotic translation initiation factor 3 subunit G-2 (*EIF3G*)	5	45 (32)	Q9VDM6	Protein biosynthesis
Eukaryotic translation initiation factor 3 subunit F-2	3	37 (32)	B4MDZ0	Protein biosynthesis
Eukaryotic translation initiation factor 3 subunit C	6	74 (32)	B4MRZ8	Protein biosynthesis
Eukaryotic translation initiation factor 3 subunit D-2	3	33 (32)	Q9VGC7	Protein biosynthesis
Eukaryotic translation initiation factor 3 subunit B (*EIF3B*)	2	46 (32)	Q0E940	Protein biosynthesis
Aminoacyl tRNA synthetase complex-interacting multifunctional protein 2	4	36 (32)	Q9VUR3	Protein biosynthesis
H. Flight behavior				
ADP, ATP carrier protein (*SLC25A4*)	3	35 (32)	Q26365	Flight behavior/response to mechanical stimulus
Protein turtle	5	35 (32)	Q967D7	Flight behavior/larval behavior
I. Others				
*Female-specific protein transformer	5	52 (32)	Q24669	Sexual differentiation
Trafficking kinesin-binding protein milt (*TRAK1*)	6	62 (32)	Q960V3	Nebenkern formation

even though the hypostomal sclerite appeared to be properly developed. No optical depression was observed in 6-day old niacin-deficient second-instar larvae, but it was observed in 4-day-old healthy second-instar larvae.

3.3 | Microarray analysis for gene expression

Among 14 008 genes, 266 and 124 were newly expressed or upregulated in the healthy larvae and the niacin-deficient larvae, respectively, whereas 170 genes were newly expressed or upregulated in both the healthy larvae and niacin-deficient larvae. The spalt-major protein gene that participates in sensory organ development or photoreceptor cell differentiation, including R8 cell differentiation, was upregulated by 1.5-fold in niacin-deficient larvae (Figure 3 and Table 1), indicating that R8 photoreceptor cell differentiation may be defective. Additionally, optomotor-blind-related gene-1 (*org-1*) was upregulated by twofold in the niacin-deficient larvae (Figure 3 and Table 3). Real-time PCR showed that expression of *org-1* in the niacin-deficient larvae increased by 17-fold relative to healthy larvae (Figure 3 and Table 3). The *org-1* gene regulates disabled protein expression (Table 3) and may play a crucial role in medfly optic lobe development [23, 24]. The

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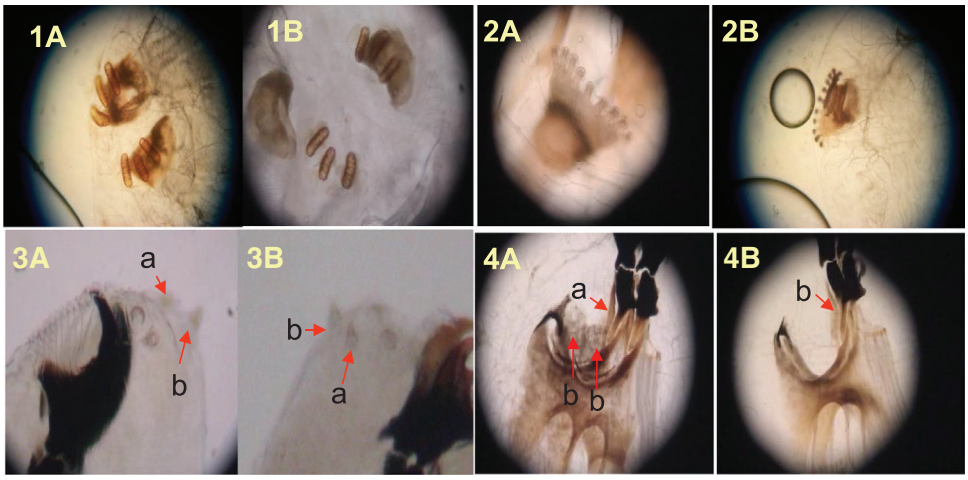

FIGURE 1 Organs of second instar *Ceratitis capitata* reared on diet with (A) and without (B) niacin supplement: (1) posterior spiracle; (2) anterior spiracle; (3) (a) aptcnnal sensory organ, (b) maxillary sensory organ; (4) (a) hypostomal sclerite, (b) ocular depression

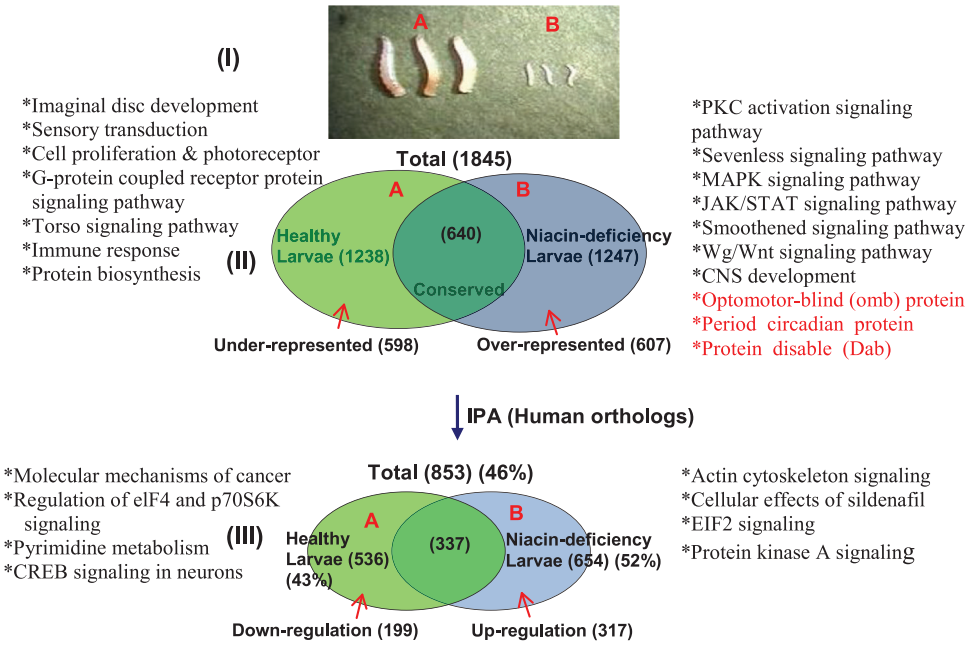

FIGURE 2 Photograph of Medfly larvae reared on a niacin-free diet fortified with 707 μg/g of nine other vitamins (I, B), number of protein identified (II), and number of proteins mapped to human ortholog via IPA (III). Venn diagrams (II) showing numbers of proteins differentially identified in the parentheses, of which 640, 598, and 607 proteins were overlapped, underexpressed, and overexpressed, respectively. Venn diagrams (III) showing 853 of the 1845 identified proteins successfully mapped by IPA (human database)

fusilli (*fus*) gene, which was upregulated in niacin-deficient larvae, is involved in the MAPK and epidermal growth factor receptor (EGFR) signaling pathways (Table 3), which is consistent with upregulation of the MAPK signaling pathway observed in the niacin-deficient larvae (Table 1). The *fus* gene acts downstream of the EGFR signaling pathway.[7] EGFR is expressed in a variety of cell types, and its aberrant activation or expression is known to cause cancer in humans.[23] The rutabaga gene controls memory, behavior, and cell communication and is related to the expression of period clock circadian protein (Table 1). CG189594, which increased by 2.3-fold in niacin-deficient larvae, is an immune-induced protein homologous to a mammalian serine kinase protease inhibitor that mediates the mitogen-activated protein kinase and NF-κB signaling pathways.[24,25]

3.4 | Signal transduction pathway in larvae fed on niacin-deficient diet

Niacin deficiency leads to aberrant activation of EGFR. The effects of niacin deficiency through EGFR in niacin-deficient larvae were proposed based on the microarray and proteomic data. Specifically, signaling through EGFR activates inositol 1, 4, 5-triphosphate receptor

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TABLE 3 Genes identified from the GeneChip *Drodophila* Genome Array with fold change >±1.3—value indicates the significance of the detection call

	Healthy larvae		Niacin-deficient larvae			
Gene name/Symbol	Signal	*P*-value	Signal	*P*-value	Fold change	Biological function
Spalt-related protein/*salr*	10.4	0.48748	29.7	.135974	+1.49	Wing vein morphogenesis regulation
Optomotor-blind-related gene-1/ *org-1*	20.1	0.586954	40.8	.198363	+2.03	Regulation of transcription from RNA polymerase II promoter mesoderm development
Fusilli/*fus*	11.6	0.364945	27.9	.088557	+1.39	EGFR signaling pathway
Tribbles/*trbl*	34.1	0.054711	49.1	.054711	+1.44	Gastrulation signal transduction
Hormone receptor-like in 38 stardust couch potato/*Hr38*	153.4	0.001755	214.9	.001432	+1.4	Regulation of transcription from RNA polymerase II promoter intracellular signaling cascade epidermis
Smarter clock Rutabaga/ *Smr Clk rut*	125.3	0.000491	235.1	.000491	+1.88	Locomotor rhythm positive regulation of transcription
Stretchin Mlck	20.9	0.135974	34.5	.135974	+1.65	Mitosis mesoderm development
CG15629	12.1	0.122747	29.6	.006575	+1.48	Metabolism visual perception
CG18594	18.8	0.122747	44.9	.296809	+2.25	Signal transduction/Kinase inhibitor activity
CG6180	20.2	0.235169	28.5	.198363	+1.41	Signal transduction/Phosphatidylethanolamine binding kinase inhibitor activity
CG32085	22.2	0.254899	43.1	.235169	+1.94	Proteolysis protein metabolism
CG320805	28.7	0.078906	16.5	.296809	−1.44	Carbohydrate metabolism, Cation transport
CG4984	29.8	0.099061	8.6	.34162	−1.49	

The array element with the lowest *P* value (<0.05) is represented when multiple elements were identified for a gene. Fold change (FC) indicates the relative change in expression levels between the experiment and baseline targets: +increased expression; −decreased expression (>±1.3). The FC was calculated as described in expression analysis metrics (p 454, Appendix E) using Affymetrix Microarray Suite 5.0 (MAS).

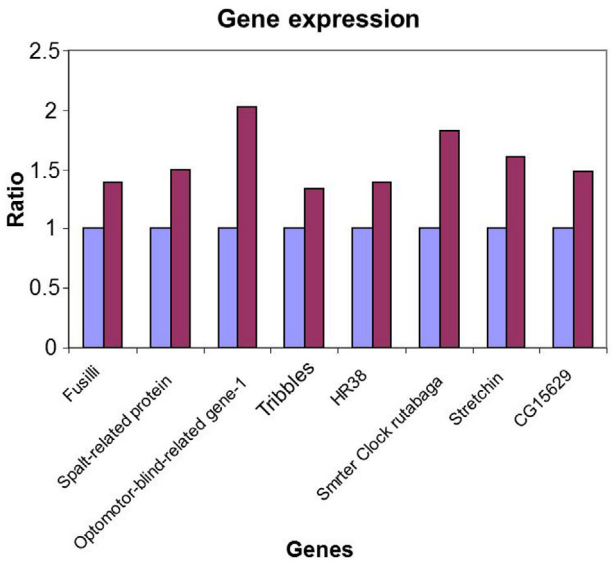

FIGURE 3 Gene expression of *org-I* by Real Time PCR (Relative Quantity: ß-actin CT Mean/sample CT Mean)

(InsP3R) followed by the Ras and Raf/MAPK pathways. It is known that Ras activates Raf, MEK, and MAPK and forms a cascade in which each kinase activates the next via phosphorylation. The C-jun-amino-terminal kinase (JNK) pathway is a Ras signaling pathway that links the signals from growth factor receptors with activation of the MAPK Kinase cascade of phosphorylation, leading to cell growth and differentiation. Raf and MAPK probably lead to overexpression of *org-1*, which plays an important role in optic lobe development, in the niacin-deficient larvae. Phosphorylated Eye-PKC, JNK-interacting protein, Rac-like GTP-binding protein, and tyrosine-protein were detected upon 2D-GE and electron transfer dissociation-LC-IT/MS, while the detected EGFR was not phosphorylated, but was oxidized. The extracellulose signal regulated kinases (ERK) Hsp 70 and phospholipase C (PLC) were detected by western blotting (data not shown). Heat shock protein (Hsp 70), which regulates the synthesis of seven proteins, is related to the MAPK signaling pathway and plays an important role in protein folding and helps to protect proteins from damage caused by stress,[24] was over expressed in niacin-deficient larvae relative to the healthy larvae. Western blotting revealed that ERK, which is related to MAPK, was overexpressed in the niacin-deficient larvae relative to the control larvae (data not shown).

4 | CONCLUSIONS

Proteomic analyses identified 1845 proteins, of which approximately one third (605) were upregulated and one third (519) downregulated in the niacin-deficient larvae relative to the healthy larvae, while the

remaining one third were conserved. The optomotor-blind protein and optomotor-blind-related gene-1 (*org-1*) were upregulated for about 18-fold in the niacin-deficient larvae, as was *Fusilli* (*fus*) gene, which both fus and org-1 genes regulate the EGFR signaling pathway. The overexpressed PKC prohibits normal eye development. The downregulated proteins in the niacin-deficient larvae included zinc finger protein 2, kinesin-like protein costa, protein apterous, carboxypeptidase D, SP460, short stop (isoform H), and protein dachsous, which are pertinent to imaginal disc-derived wing/leg morphogenesis. In conclusion, dietary niacin deficiency affects the eye development of ocular depression and the expression of *org-1* gene, leading to the abnormal wing development in the medfly larvae.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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