Appendix A

Proteomic and metabolomic analysis reveals rapid and extensive nicotine detoxification ability in honey bee larvae

Esther E. du Rand [a,b]*, Hannelie Human[b], Salome Smit[c], Mervyn Beukes[a], Zeno Apostolides[a], Susan W. Nicolson[b] and Christian W.W. Pirk[b]

[a]Department of Biochemistry, University of Pretoria, Private Bag X20, Hatfield 0028, South Africa
[b]Department of Zoology and Entomology, University of Pretoria, Private Bag X20, Hatfield 0028, South Africa
[c]Proteomics Unit, Central Analytical Facility, Stellenbosch University, Private Bag X1, Matieland 7602, South Africa

Supplementary Materials and Methods

Supplementary Tables

Supplementary Table 1. Significantly up-regulated proteins in honey bee larvae after 72 h of nicotine exposure (ANOVA t-Test, $p \leq 0.05$; $q \leq 0.01$).

Beebase Identifier	Accession	Fold change	p-value	Homologous Functions	Biological process/Function
Energy metabolism					
GB17255	XP_003250793.1	4	0.0140	ATP synthase subunit delta, mitochondrial isoform 2	Oxidative phosphorylation
GB40305	XP_397330.3	2	0.0388	NADH dehydrogenase [ubiquinone] iron-sulfur protein 2, mitochondrial isoform 1	Oxidative phosphorylation
GB10474	XP_001120610.1	2	0.0100	hypothetical protein LOC725881 isoform 1	Oxidative phosphorylation
GB45153	XP_391916.2	3	0.0058	NADH dehydrogenase [ubiquinone] flavoprotein 1, mitochondrial	Oxidative phosphorylation
GB49306	XP_625078.1	2	0.0009	ATP synthase subunit gamma, mitochondrial	Oxidative phosphorylation
GB42482	XP_396263.4	2	0.0270	v-type proton ATPase 116 kDa subunit a isoform 1-like isoform 1	Oxidative phosphorylation
Carbohydrate metabolism					
GB44897	XP_624349.1	2	0.0484	UDP-N-acetylhexosamine pyrophosphorylase, *mmy*	Amino sugar and nucleotide sugar metabolism
	XP_396639.1	2	0.0066	NADH-cytochrome b5 reductase 2-like isoform 1	Amino sugar and nucleotide sugar metabolism
GB54391	XP_624707.1	2	0.0008	putative glycogen [starch] synthase-like	Glycogen synthesis
GB46214	XP_625114.1	2	0.0099	phosphoglycerate mutase 2-like	Glycolysis
GB52074	XP_625090.2	2	0.0126	6-phosphogluconate dehydrogenase, decarboxylating	Pentose phosphate pathway
GB55489	NP_001106141.1	3	0.0172	trehalase precursor	Starch and sucrose metabolism
GB52753	XP_393056.2	2	0.0157	succinate dehydrogenase cytochrome b560 subunit, mitochondrial, Sdh	TCA cycle
GB48527	XP_392811.2	2	0.0002	probable isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial-like	TCA cycle
GB44983	XP_003250858.2	2	0.0211	dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex, mitochondrial-like	TCA cycle

Beebase Identifier	Accession	Fold change	p-value	Homologous Functions	Biological process/Function
GB49967	H9KJR9	3	0.0066	Malic enzyme, Mdh	TCA cycle
Detoxification and stress response					
GB42809	XP_395299.2	2	0.0009	translationally-controlled tumor protein homolog isoform 1 (Tctp)	Antioxidant activity
GB48215	H9KQH7	2	0.0056	thioredoxin 1-like 3	Antioxidant activity
GB48574	XP_003250408.1	3	0.0335	thioredoxin-2 isoform 1	Antioxidant activity
GB49471	XP_392870.1	2	0.0075	glutaredoxin 3	Antioxidant activity
GB55561	XP_001121022.1	2	0.0022	copper transport protein ATOX1-like	Antioxidant activity
GB40718	NP_001171496.1	3	0.0048	thioredoxin reductase 1 isoform 1	Cell redox homeostasis
	XP_395447.4	2	0.0140	probable ubiquitin carboxyl-terminal hydrolase FAF-X	Cell stress response
GB50652	XP_393315.2	2	0.0390	t-complex protein 1 subunit epsilon, Cct5	Chaperone proteins, folding actin and tubulin
GB54343	XP_624910.1	2	0.0068	10 kDa heat shock protein, mitochondrial-like	Chaperone proteins, functions as a co-chaperone with Hsp 60
GB12215	XP_001119835.1	2	0.0173	dnaJ homolog subfamily A member 1	Chaperone proteins, the DnaJ family (Hsp 40 family) are co-chaperones of Hsp 70
GB43690	XP_001123053.2	2	0.0027	hypothetical protein LOC727344, nitrile-specifier protein	Detoxification, function as cofactor to proteins involved in secondary metabolite metabolism
GB47279	XP_006564499.1	2	0.0283	cytochrome P450 6k1, CYP6BD1	Detoxification
GB53134	XP_003249371.1	2	0.0030	multidrug resistance-associated protein 1-like	Detoxification
GB19820	XP_393971.1	2	0.0079	cytochrome P450 9e2, CYP9Q1	Detoxification
	XP_624702.2	2	0.0046	ubiquitin carboxyl-terminal hydrolase 5-like	Positive regulation of proteasomal ubiquitin-dependent protein catabolic process
Lipid metabolism					
	XP_001120449.2	2	0.0488	fatty acyl-CoA reductase 1-like	Biosynthesis of wax
GB51247	XP_397228.2	1	0.0027	elongation of very long chain fatty acids protein AAEL008004-like	Fatty acid biosynthesis
GB47140	XP_006558238	3	0.0244	hypothetical protein LOC409465 isoform 1, Lsd-1	Lipid storage
Growth and development related					
GB50482	H9K5Z0	3	0.0333	hypothetical protein LOC72497, SP23	Chitin metabolism
GB42597	NP_001167611.1	2	0.0301	cuticular protein	Component of cuticle
	NP_001257753.1	2	0.0062	flexible cuticle protein 12, CPR12	Component of cuticle
GB13601	NP_001257760.1	2	0.0008	pupal cuticle protein C1B	Component of the cuticle of the pupa; structural constituent of cuticle

Beebase Identifier	Accession	Fold change	p-value	Homologous Functions	Biological process/Function
	XP_393853.1	2	0.0212	slit homolog 1 protein-like	Development; axon guidance; differentiation, neurogenesis
GB40770	XP_001120613.2	2	0.0148	dehydrogenase/reductase SDR family member 11-like	Growth and development
GB51696	NP_001092187.1	2	0.0216	hexamerin 70c	Nutrient storage
	XP_393878.3	2	0.0034	IGF-II mRNA-binding protein	Nutrient storage
GB52324	NP_001011583.1	2	0.0449	chemosensory protein 3 precursor, ASP3c	Odorant binding protein; cuticle maturation
GB15582	XP_006559884.1	2	0.0265	14-3-3 protein epsilon isoform 1	Signalling pathways
Protein processing (including folding, sorting, modification, transport)					
	XP_393328.2	2	0.0377	coatomer subunit epsilon, cope	Biosynthetic protein transport from the ER, via the Golgi up to the trans Golgi network.
	XP_393370.3	2	0.0460	26S proteasome non-ATPase regulatory subunit 12, partial	Folding, sorting and degradation; Proteasome
	XP_392515.2	2	0.0003	protein transport protein Sec23A-like isoform 1	Intracellular protein transport, ER to Golgi vesicle-mediated transport
	XP_396270.4	2	0.0040	importin-4-like	Intracellular protein transport
	XP_395332.2	2	0.0225	exportin-2, Cas	Intracellular protein transport
	XP_396469.1	2	0.0009	exportin-1, emb	Intracellular protein transport
	XP_395549.2	3	0.0021	coatomer subunit gamma isoform 1, gammaCop	Intracellular trafficking and secretion
	XP_624433.2	2	0.0011	dolichyl-diphosphooligosaccharide-protein glycosyltransferase 48 kDa subunit-like isoform 1	Protein modification, glycosylation
GB46512	XP_624548.2	2	0.0241	AFG3-like protein 2-like	Protein processing
	XP_001120162.1	2	0.0081	endoplasmic reticulum resident protein 29-like	Protein processing in ER
	XP_623191.2	2	0.0387	protein ERGIC-53-like isoform 1	Protein processing in ER
	XP_625125.2	2	0.0011	glucosidase 2 subunit beta-like	Protein processing in ER; deglucosylation
	XP_623111.3	2	0.0011	clathrin heavy chain-like isoform 1	Protein transport
	XP_623555.2	2	0.0176	nascent polypeptide-associated complex subunit alpha-like isoform 1	Protein transport; prevents inappropriate targeting of non-secretory polypeptides to the ER
Transcription					
	XP_623333.1	2	0.0225	splicing factor 3B subunit 3 isoform 1	mRNA processing, mRNA splicing
	XP_001120188.1	2	0.0005	u6 snRNA-associated Sm-like protein LSm2-like	mRNA processing; RNA degradation
	XP_393330.2	2	0.0023	regulator of nonsense transcripts 1, Upf1	mRNA surveillance pathway, RNA transport
	XP_395300.3	2	0.0321	la protein homolog	RNA processing

Beebase Identifier	Accession	Fold change	p-value	Homologous Functions	Biological process/Function
	XP_001120479.1	2	0.0204	ribonuclease kappa-like	rRNA transcription (endonuclease activity)
	XP_623668.3	2	0.0096	ATP-dependent RNA helicase p62-like isoform 1	Spliceosome
	XP_624498.2	2	0.0493	peptidyl-prolyl cis-trans isomerase FKBP1A	Spliceosome
	XP_624014.2	2	0.0149	pre-mRNA-processing-splicing factor 8-like	Spliceosome
	XP_623732.1	2	0.0245	splicing factor 3B subunit 1-like isoform 1	Spliceosome; mRNA processing, mRNA splicing
	XP_001122345.2	1	0.0387	transcription factor BTF3 homolog 4-like	Transcription
	XP_624197.3	2	0.0071	elongator complex protein 1-like	Transcription
	XP_396542.4	2	0.0414	serrate RNA effector molecule homolog	
Translation					
	XP_623301.1	2	0.0244	arginyl-tRNA synthetase, cytoplasmic	Aminoacyl-tRNA biosynthesis
	XP_395921.4	2	0.0140	hypothetical protein LOC412464	mRNA transport
	XP_396588.2	2	0.0066	60S ribosomal protein L15	Ribosome
	XP_001121282.1	2	0.0235	60S ribosomal protein L37a	Ribosome
	XP_393034.1	3	0.0097	60S ribosomal protein L7a	Ribosome
	XP_393092.2	2	0.0256	60S ribosomal protein L10 isoform 1	Ribosome
	XP_001121184.1	3	0.0289	hypothetical protein LOC725324	Ribosome biogenesis in eukaryotes
	XP_623414.3	3	0.0077	midasin-like	Ribosome biogenesis in eukaryotes
	XP_624378.1	2	0.0253	rRNA 2'-O-methyltransferase fibrillarin	Ribosome biogenesis in eukaryotes
	XP_624438.1	2	0.00698	>eukaryotic translation initiation factor 3 subunit L-like	Translation; RNA transport
	XP_396174.4	2	0.0299	nuclear pore complex protein Nup155	Translation; RNA transport
	XP_003251878.1	2	0.0084	eukaryotic translation initiation factor 2 subunit 2-like	Translation; RNA transport
	XP_003251437.1	3	0.0200	eukaryotic initiation factor 4A-like	Translation; RNA transport
	NP_001011628.1	2	0.0033	elongation factor 1-alpha	Translation; RNA transport
	XP_395387.4	2	0.0228	nuclear pore complex protein Nup205	Translation; RNA transport
Other proteins					
	XP_625229.1	2	0.0042	catenin alpha	Cell adhesion cytoskeleton
	XP_001123160.2	2	0.0141	sorting nexin-6-like	Cell communication
	XP_395776.1	2	0.0029	ran-specific GTPase-activating protein-like	Cell cycle; intracellular signalling
	XP_393642.4	3	0.0003	adenylyl cyclase-associated protein 1-like isoform 1	Cell morphogenesis

Beebase Identifier	Accession	Fold change	p-value	Homologous Functions	Biological process/Function
	XP_623438.1	3	0.0215	dihydrolipoyl dehydrogenase, mitochondrial-like isoform 1	Cell redox homeostasis
	XP_625092.3	2	0.0477	conserved oligomeric Golgi complex subunit 2-like	Golgi structure and function; vesicular trafficking
	XP_623361.3	2	0.0088	guanylate cyclase 32E-like	Intracellular signal transduction; cGMT biosynthesis
GB51087	XP_625042.2	2	0.0143	pyridoxal kinase-like	Metabolism of cofactors and vitamins; Vitamin B6 metabolism
	XP_624452.3	2	0.0218	hypothetical protein LOC552071	Mitochondrial biogenesis
GB47407	NP_001011609	7	0.0033	histone H4	Nucleosome assembly
	XP_001120889.1	9	0.0008	histone H2B	Nucleosome assembly; core component of nucleosome.
GB48022	XP_623300.1	2	0.0339	protein henna-like isoform 2, also known as phenylalanine-4-hydroxylase	Phenylalanine, tyrosine, tryptophan metabolism
	XP_393127.3	1	0.0320	trypsin-1	Protease
	XP_393167.4	2	0.0221	protein tyrosine phosphatase type IVA 1	Signal transduction
	XP_624357.1	2	0.0022	calumenin, scf	Signal transduction, diverse superfamily of calcium sensors and calcium signalling modulators
GB41843	XP_392681.2	2	0.0268	succinyl-CoA:3-ketoacid-coenzyme A transferase 1, mitochondrial-like	Valine, leucine and isoleucine degradation
GB51088	XP_391840.3	2	0.0423	isovaleryl-CoA dehydrogenase, mitochondrial-like	Valine, leucine and isoleucine degradation
GB43905	XP_395784.2	2	0.0267	stomatin-like protein 2-like isoform 1	
GB43006	XP_392145.2	2	0.0012	glucose dehydrogenase [acceptor]	Glycine, serine and threonine metabolism
	XP_003249222.1	3	0.0298	calerythrin isoform 2, *Scp2*	
	XP_397351.3	2	0.0031	constitutive coactivator of PPAR-gamma-like protein 1 homolog isoform 1	
	XP_001122466.1	2	0.0012	HIG1 domain family member 2A-like	
GB40157	XP_623685.1	2	0.0045	hypothetical protein LOC408421 isoform 2	
	XP_391992.4	2	0.0239	hypothetical protein LOC408444	
	XP_623180.1	2	0.0138	hypothetical protein LOC408592 isoform 2	
	XP_625245.1	3	0.0186	hypothetical protein LOC551318	
	XP_001120943.2	2	0.0087	hypothetical protein LOC725047	
	XP_395159.2	2	0.0076	phosphotriesterase-related protein-like	

Beebase Identifier	Accession	Fold change	p-value	Homologous Functions	Biological process/Function
	XP_624431.2	2	0.0060	protein DDI1 homolog 2-like	
	XP_001121381.1	2	0.0004	protein FAM151B-like	
	XP_392962.1	2	0.0082	receptor of activated protein kinase C 1, Rack1	
	XP_392222.3	2	0.0249	sequestosome-1	
	XP_003249921.1	2	0.0011	transmembrane protein 214-like	
	H9K3Q4	1	0.0480	uncharacterised protein	
	H9KGG6	1	0.0067	uncharacterised protein	
	H9KPB9	2	0.0358	uncharacterised protein	
	H9K047	3	0.0415	uncharacterised protein	
	H9KR51	2	0.0292	uncharacterised protein, LOC408532	
	H9K7J8	1	0.0177	uncharacterised protein	
	H9JZ46	1	0.0098	uncharacterised protein	
	H9KH00	2	0.0284	uncharacterised protein	
	H9KKF7	1	0.0330	uncharacterised protein	
	H9K046	1	0.0010	uncharacterised protein	
	H9JZ44	2	0.0150	uncharacterised protein	
	H9KNY1	1	0.0067	uncharacterised protein	
	H9KQI7	2	0.0115	uncharacterised protein	
	H9KD88	3	0.0344	uncharacterised protein	
	H9KB60	3	0.0013	uncharacterised protein	
	H9K7Z6	1	0.0318	uncharacterised protein	
	H9JZH7	1	0.0066	uncharacterised protein	

Supplementary Table 2. Significantly down-regulated proteins in honey bee larvae after 72 h of nicotine exposure (ANOVA t-Test, $p \leq 0.05$; $q \leq 0.01$).

Beebase Identifier	Accession	Fold change	p-value	Homologous Functions	Biological process/Function
Genetic information processing					
	XP_393883.3	2	0.0008	proteasome activator complex subunit 3-like	Folding, sorting and degradation; Proteasome
	XP_395743.2	2	0.0339	leucyl-tRNA synthetase, cytoplasmic-like isoform 1	Regulation of translational fidelity (protein biosynthesis)
	XP_394825.2	2	0.0320	elongation factor G, mitochondrial-like isoform 1	Transcription
	XP_003251532.1	1	0.0221	enhancer of yellow 2 transcription factor	Transcription regulations, mRNA transport
	XP_003249340.1	3	0.0022	LOC409479, Ribosomal protein, RpL6	Translation
	XP_624438.1	2	0.0268	eukaryotic translation initiation factor 3 subunit L-like	Translation
	XP_001121387.1	2	0.0423	eukaryotic peptide chain release factor subunit 1-like	Translation
	XP_001123355.2	2	0.0267	acylamino-acid-releasing enzyme-like	Translation
	XP_395202.3	2	0.0298	valyl-tRNA synthetase isoform 1	Translation; Aminoacyl-tRNA biosynthesis
	XP_393322.1	2	0.0031	60S ribosomal protein L18a, RpL18A	Translation; Ribosome
	XP_393034.1	3	0.0012	60S ribosomal protein L7a	Translation; Ribosome
	XP_394949.3	2	0.0045	nucleoporin NUP188 homolog	Translation; RNA transport
Lipid metabolism					
	XP_394614.1	3	0.0239	putative aldehyde dehydrogenase family 7 member A1 homolog isoform 2	Fatty acid beta-oxidation
	XP_393211.4	3	0.0138	short/branched chain specific acyl-CoA dehydrogenase, mitochondrial-like	Fatty acid beta-oxidation
	XP_392111.2	2	0.0186	probable medium-chain specific acyl-CoA dehydrogenase, mitochondrial-like	Fatty acid beta-oxidation
Energy metabolism					
	XP_001122467.1	2	0.0087	cytochrome b-c1 complex subunit 8-like	Oxidative phosphorylation
	XP_396465.1	2	0.0076	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 5, Ndufa5	Oxidative phosphorylation
	XP_623441.1	2	0.0060	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 6-like	Oxidative phosphorylation

Beebase Identifier	Accession	Fold change	p-value	Homologous Functions	Biological process/Function
Carbohydrate metabolism					
	XP_623403.2	2	0.0004	hexokinase type 2-like	Glycolysis
	NP_001035326.1	2	0.0082	alpha glucosidase 2; hbg1	Starch and sucrose metabolism; Essential for the degradation of glycogen to glucose
	XP_001120125.2	2	0.0185	fumarate hydratase, mitochondrial-like	TCA cycle
Other proteins					
	XP_395840.2	2	0.0011	excitatory amino acid transporter 3, Eaat-3	Amino acid metabolism and transport
	XP_397119.2	2	0.0480	serine-pyruvate aminotransferase, mitochondrial-like	Amino acid metabolism and transport
	XP_624758.3	3	0.0067	neutral and basic amino acid transport protein rBAT isoform 2, AGLU-like 2	Amino acid metabolism and transport
	XP_393339.3	2	0.0358	fasciclin-2 isoform 1, IGFn3-12	Cell adhesion
	NP_001171499.1	2	0.0010	glutathione S-transferase D1, GstD1	Detoxification or oxidative stress response
	XP_001120951.2	2	0.0078	gamma-glutamylcyclotransferase-like	Glutathione metabolism
	XP_396480.3	2	0.0097	serine/threonine-protein kinase OSR1-like	Oxidative stress response kinase
	NP_001164444.1	2	0.0127	peroxiredoxin-like protein, Tpx-4	Oxidative stress response; Cellular Processes, Transport and catabolism, Peroxisome
	XP_393694.4	4	0.0132	four and a half LIM domains protein 2, Lmpt	Protein-protein signalling
	XP_001119959.2	2	0.0391	bleomycin hydrolase-like	Proteolysis
	H9K2W4	2	0.0171	uncharacterized protein	Unknown
	H9KKY0	1	0.0092	uncharacterized protein	Unknown
	H9K1C8	1	0.0375	uncharacterized protein, LOC727029	Unknown
	H9KPB2	1	0.0022	uncharacterized protein	Unknown
	NP_001011580.1	6	0.0013	major royal jelly protein 2 precursor	Unknown
	XP_396314.2	2	0.0318	protein msta, isoform B-like	Unknown
	NP_001014430.1	2	0.0066	troponin C type I	Unknown
	XP_624843.2	2	0.0339	filamin-A-like	Unknown
	H9K0R5	1	0.0088	uncharacterized protein	Unknown
	XP_003251320.1	3	0.0143	PRA1 family protein 3-like isoform 1	Unknown
	XP_392775.4	2	0.0218	hypothetical protein LOC409252	Unknown
	NP_001127805.1	2	0.0033	hypothetical protein LOC100187599	Unknown

Supplementary Table 3. The metabolic fate of nicotine in *A. mellifera* larvae. Nicotine and its metabolites detected in larvae after consuming a 50 ppm nicotine diet for 72 h. Data represent the mean of six experiments ± SE and are expressed as ppm detected and percentage of the total nicotine and nicotine metabolites detected (n=6; 50 larvae per sample).

Compound	ppm	%
Nicotine	0.03 ± 0.01	1.4
Cotinine N-oxide	0.04 ± 0.01	2.1
Cotinine	0.1 ± 0.03	5.3
4-Hydroxy-4-(3-pyridyl) butanoic acid	1.6 ± 0.28	91.1

Supplementary Table 4. MRM transitions and ion optic parameters for nicotine and nicotine metabolites.

Compound	Cone voltage (V)	Collision Energy (V)	ES^+ MS transition $(m/z)^+$
Nicotine	25	20	163.0 → 130.1
Nicotine	25	26	163.0 → 117.0
Nicotine	25	15	163.0 → 132.1
Nornicotine	25	18	149.2 → 132.1
Nicotine N-oxide	25	22	179.3 → 132.1
Cotinine	15	20	177.2 → 79.8
Cotinine	15	15	177.2 → 146.0
Norcotinine	25	15	149.2 → 146.1
Cotinine N-oxide	25	15	193.2 → 162.0
3'-Hydroxy-cotinine	25	15	193.3 → 134.0
4-Hydroxy-4-(3-pyridyl) butanoic acid	25	18	182.2 → 164.0
Nicotine-glutathione	25	12	339.2 → 163.1
Cotinine-glutathione	25	12	353.1 → 177.1
3'-Hydroxy-cotinine-glutathione	25	12	369.0 → 193.0

Supplementary Materials and Methods

Metabolomic profiling.

Sample preparation. Samples of 50 bee larvae were lyophilized and ground with a pestle and mortar. Using an automated MicroLab STAR® liquid handler (Hamilton, Salt Lake City, UT, USA), protein was precipitated from the samples using a series of aqueous extractions optimized for maximum recovery of small molecules (Metabolon Inc., Durham, NC, USA). The resulting extract was split into equal aliquots for liquid chromatography (LC) and gas chromatography (GC) analysis, respectively. The sample aliquots were placed briefly on a TurboVap® (Zymark Corp., Hopkinton, MA, USA) to remove any residual organic solvent and subsequently frozen and dried under vacuum. The LC sample aliquots were reconstituted in either 0.1% formic acid or 6.5 mM ammonium bicarbonate (pH 8). The GC sample aliquots were derivatized using bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) at 60 °C for 1 h. All samples were spiked with injection standards at fixed concentrations.

Mass spectrometry. Non-targeted metabolic profiling was performed using three independent platforms (Evans et al., 2009). Ultrahigh performance liquid chromatography/tandem mass spectrometry (UPLC/MS/MS^2) optimized for acidic species, UPLC/MS/MS^2 optimized for basic species, and gas chromatography/mass spectrometry (GC/MS) were performed. The LC/MS portion of the platform was based on a Waters ACQUITY UPLC and a Thermo-Finnigan linear trap quadrupole (LTQ) mass spectrometer, which consisted of an electrospray ionization (ESI) source and linear ion-trap (LIT) mass analyzer. In two independent injections using separate dedicated columns, one sample aliquot was analyzed using optimized acidic conditions with the MS operating in positive ion detection mode and the other sample aliquot was analyzed using optimized basic conditions with the MS operating in negative ion detection mode. Sample aliquots reconstituted in acidic conditions were gradient eluted using water and methanol containing 0.1% formic acid, while the basic sample aliquots were eluted with water and methanol containing 6.5 mM ammonium bicarbonate. The MS analysis alternated between MS and data-dependent MS^2 scans using dynamic exclusion. For GC/MS analysis a 5% phenyl column and a temperature ramp of 40 to 300 °C over a 16 min period was used. Samples were

analyzed on a Thermo-Finnigan Trace DSQ fast-scanning single-quadrupole mass spectrometer using electron impact ionization.

Data analysis. Metabolites were identified by comparing the ion features in the samples to the entries of purified standards in a metabolomic reference library (Evans et al., 2009) that includes retention time, molecular weight (*m/z*), preferred adducts, in-source fragments as well as associated mass spectra. General biochemical pathways, as illustrated in the Kyoto Encyclopaedia of Genes and Genomes server (KEGG) (Kanehisa et al., 2014) were used to assign identified metabolites to specific metabolic networks in order to identify any overrepresented pathways. Missing values for a given metabolite were assigned the observed minimum value (minimum value imputation). Raw area counts for each compound were re-scaled by dividing each sample value by the median value for the specific metabolite in order to visualize the data more conveniently. R (http://cran.r-project.org) was used for the statistical analysis of the data. A log transformation was applied to the observed relative abundances and the fold change was calculated for each metabolite identified as the means ratio of the control and treatment groups. Welch's two-sample Student t-Tests (p-value<0.05) were used to determine whether or not each metabolite significantly increased or decreased in abundance. The false discovery rate (FDR) was calculated to correct for multiple Welch's two-sample Student t-Test comparisons for the hundreds of compounds detected. The FDR for a given set of metabolites is estimated by the Q-value (Storey, 2002). A Q-value smaller than 0.10 was used as an indication of high confidence in a result (Storey, 2002). Other lines of evidence were also taken into consideration when the Q-value exceeded 0.10, such as if the metabolite shares a common pathway with a highly significant compound, or if the metabolite is in a similar biochemical functional family with other significant compounds.

Proteomic profiling.

Sample Preparation. Fifteen bee larvae per sample were homogenized in 1 ml of lysis buffer using an Ultra-Turrax® homogenizer (IKA®-Werke GmbH & Co. KG, Staufen, Germany). The lysis buffer consisted of 7 M urea, 2 M thiourea, 4% CHAPS, 1% DTT, 40 mM Tris and Complete Protease Inhibitor tablets (Roche Diagnostics, Mannheim, Germany). After homogenization the samples were sonicated on ice using a Sonifier® Cell Disrupter B-30 fitted

with a microtip (Branson Ultrasonics Corporation, Danbury, CT, USA) in 4 x 15 s pulses with 10 s cooling in between (settings: pulsed; Output control 3; % Duty cycle 60). Subsequently, the samples were centrifuged at 14 000 x *g* for 10 min. Trichloroacetic acid was added to the collected supernatants to a final concentration of 10%, followed by incubation on ice for 30 min to ensure the precipitation of proteins and desalting. The precipitated proteins were collected by centrifugation at 14 000 x *g* for 10 min. The collected pellets were washed three times with ice cold acetone. The washed pellets were dried and solubilized in 7 M urea, 2 M thiourea and 100 mM Tris (pH 8) before being stored at -80 °C. Protein concentrations were determined using the Coomassie Plus™ (Bradford) Assay Kit (Pierce, Rockford, IL, USA).

Electrophoresis and in gel trypsin digestion. Extracted proteins were resolved on precast 10-well 12% Mini-Protean TGX Stain Free gels (Bio-Rad, Hercules, CA) according to the manufacturer's instructions (55 µg protein were loaded per lane). Coomassie® Brilliant Blue G250 (Merck, Darmstadt, Germany) was used to visualize the protein bands. After visualization, each sample lane was cut into 4 pieces. All gel pieces were cut into smaller cubes and washed twice with water followed by a 10 min washing step using 50% acetonitrile. The acetonitrile was replaced with 50 mM ammonium bicarbonate and incubated for 10 min. Gel pieces were then incubated in 100% acetonitrile until they turned white and were then dried *in vacuo*. Proteins were reduced with 10 mM DTT for 1 h at 57 °C. This was followed by brief washing steps of ammonium bicarbonate followed by 50% acetonitrile before the proteins were alkylated with 55 mM iodoacetamide for 1 h in the dark. Following alkylation, the gel pieces were washed with ammonium bicarbonate for 10 min and then 50% acetonitrile for 20 min, before being dried *in vacuo.* The gel pieces were digested with 100 µl of a 10 ng/µl trypsin solution at 37 °C overnight. The resulting peptides were extracted twice with 70% acetonitrile containing 0.1% trifluoroacetic acid for 30 min followed by a 30 min extraction step using 100% acetonitrile before being dried. The dried peptides were then dissolved in 5% formic acid and cleaned using Stage Tips (Thermo Scientific, IL, USA) according to the instructions. The peptides were dried and stored at -20 °C. Dried peptides were dissolved in 5% acetonitrile in 0.1% formic acid and 10 µl injections were made for nano-LC chromatography.

Mass spectrometry. All experiments were performed on a Thermo Scientific EASY-nLC II connected to a LTQ Orbitrap Velos mass spectrometer (Thermo Scientific, Bremen, Germany) equipped with a nano-electrospray source. For liquid chromatography, separations were carried

on an EASY-Column (2 cm, ID 100µm, 5 µm, C18) pre-column followed by a X-Bridge BEH130 NanoEase column (15 cm, ID 75 µm, 3.5 µm, C18). Mobile phase A consisted of 0.1 (v/v) formic acid and mobile phase B consisted of 90% (v/v) acetonitrile and 0.1% (v/v) formic acid. The gradient used was: 0-5 min, 5-17% B; 5-95 min, 17-25% B; 95-105 min, 25-60% B; 105-110 min 60-80% B; 110-120 min 80% B. The flow rate was set at 300 nl/min. The mass spectrometer was operated in data-dependent mode to automatically switch between Orbitrap-MS and LTQ-MS/MS acquisition. Data were acquired using the Xcaliber software package. The precursor ion scan MS spectra (*m/z* 400 – 2000) were acquired in the Orbitrap with resolution R = 60000 with the number of accumulated ions being 1×10^6. The 20 most intense ions were isolated and fragmented in the linear ion trap (number of accumulated ions 1.5×10^4) using collision induced dissociation. The lock mass option (polydimethylcyclosiloxane; *m/z* 445.120025) enabled accurate mass measurement in both the MS and MS/MS modes. In data-dependent LC-MS/MS experiments, dynamic exclusion was used with 60 s exclusion duration. Mass spectrometry conditions were 1.8 kV, capillary temperature of 250 °C, with no sheath and auxiliary gas flow. In the MS/MS mode, the ion selection threshold used was 500 counts and the activation Q-value was set at 0.25 and the activation time at 10 ms.

Data analysis. MaxQuant 1.2.2.5 was used to identify proteins via automated identification of tandem mass spectra against the Beebase and Uniprot *Apis melifera* databases. Carbamidomethyl cysteine was set as fixed modification. Pyro-Gln, Pyro-Glu, oxidized methionine, N-acetylation and deamidation (NQ) were set as variable modifications. The precursor mass tolerance was set to 20 ppm, and fragment mass tolerance set to 0.8 Da. Two missed tryptic cleavages were allowed. Proteins were considered positively identified when they were identified with at least 1 tryptic peptide per protein and FDR of 0.01 (protein and peptide). Statistical analysis was performed using Perseus. Raw data were transformed (log 2) and imputated (width 0.3, Down shift 1.8) to replace missing values. Two-sample ANOVA (p-value<0.05) was used to determine whether or not a protein significantly increased or decreased in abundance with at least 2-fold. Global protein expression profiles were further analysed using the KEGG (Kanehisa et al., 2014) server.

References

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