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FEBS Letters 582 (2008) 2916–2922

Metabolomic profiling of *Drosophila* using liquid chromatography Fourier transform mass spectrometry

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Received 21 May 2008; revised 12 July 2008; accepted 14 July 2008

Available online 25 July 2008

Edited by Lukas Huber

Abstract **Hydrophilic interaction chromatography (HILIC) interfaced with an Orbitrap Fourier transform mass spectrometer (FT-MS) was used to carry out metabolomic profiling of the classical *Drosophila* mutation, *rosy* (*ry*). This gene encodes a xanthine oxidase/dehydrogenase. In addition to validating the technology by detecting the same changes in xanthine, hypoxanthine, urate and allantoin that have been reported classically, completely unsuspected changes were detected in each of the tryptophan, arginine, pyrimidine and glycerophospholipid metabolism pathways. The rosy mutation thus ramifies far more widely than previously detected.**
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Keywords: Metabolomics; Fourier transform mass spectrometry; *Drosophila*

1. Introduction

Of the metazoans, *Drosophila melanogaster* offers perhaps the best trade-off between genetic tractability, availability of well-characterized genetic mutant stocks, and organismal complexity [1,2] and is thus ideal for metabolomic studies. Some mutations in metabolic pathways have been studied for nearly a century; and interactions with, and epistatic interactions between, similar mutations, established.

The *ry* mutant was originally identified as an eye colour mutation and was found to encode xanthine oxidase/dehydrogenase, an enzyme in the purine metabolism pathway [3]. *Ry* mutants accumulate the enzyme's substrates, xanthine and 2-amino-4-hydroxypteridine as larvae plus hypoxanthine in the adult; xanthine forms calculi in the malpighian tubules, bloating and distorting them [4]. The *ry* mutants lack the corresponding enzyme products uric acid and isoxanthopterin [5]. Remarkably, *ry* mutations exactly recapitulate xanthinuria type I, the renal disease caused by mutation of the human homologue of *ry*, across over 400 M years of divergent evolution [6].

Recently work was published indicating that the xanthine oxidase gene is necessary for mediating the effects of juvenile hormone (JH). In the absence of xanthine oxidase the cuticle of the insect did not develop properly when stimulated by JH [7]. As we will demonstrate in this paper the *ry* mutation ramifies into metabolic pathways remote from purine metabolism which may be required for cuticle development.

Mass spectrometry is being increasingly applied to metabolomics [8–11]. The Orbitrap mass spectrometer offers similar performance to classical FT-MS but without the requirement for a high strength magnetic field [12] and it is readily coupled to chromatographic systems. The *ry* mutation provides an excellent model to test this technology.

2. Materials and methods

2.1. Chemicals

HPLC grade acetonitrile and water were obtained from VWR International Ltd. (Lutterworth, UK). AnalaR grade formic acid (98%) was obtained from BDH-Merck (Dorset, UK). Metabolite standards were obtained from Sigma Aldrich (Dorset, UK).

2.2. Drosophila stocks

Drosophila melanogaster were kept in vials of standard medium at 25 °C and 55% r.h. on a 12:12 photoperiod. Stocks used were Canton S wild-type flies, and ry^{506} homozygotes (Bloomington Stock Center). Seven day old adult flies were anaesthetized by chilling on ice and then ground whole into 50% methanol:50% water. Ten flies were found to provide ample experimental material. Sexes were kept separate for the analysis. Following extraction, samples were stored at −24 °C until the day of analysis. Immediately prior to analysis samples were brought to room temperature, shaken and then centrifuged for 5 min at 3500 rpm and 180 μl portions of each sample were withdrawn and transferred to a chromatographic vial.

2.3. LC–MS method

LC–MS data were acquired using a LTQ Orbitrap instrument (Thermo Fisher Scientific, Hemel Hempstead, UK) set at 30 000 resolution. Sample analysis was carried out under positive ion mode. The mass scanning range was *m*/*z* 50–1200 and the capillary temperature was 200 °C. The LC–MS system was run in binary gradient mode. Solvent A was 0.1% v/v formic acid/water and solvent B was acetonitrile containing 0.1% v/v formic acid; the flow rate was 0.3 ml/min. A ZIC-HILIC column 5 μm 150 × 4.6 mm (HiChrom, Reading, UK) was used for all analyses. The gradient was as follows: 80% B (0 min) – 70% B (12 min) – 70% B at 22 min – 80% B at 30 min. The injection volume was 25 μl. Mass measurement was externally calibrated just before commencing the experiment, and was internally calibrated by lock masses. Background ions at *m*/*z* 180.10505 ($C_6H_{18}NO_3Si$) and *m*/*z* 81.52057 ($C_6H_9N_3Ca$) were used as lock masses.

2.4. Data processing

Data pre-treatment was performed using Excel-Visual basic application (VBA) subroutines (Microsoft Excel-2007) developed in-house. Multivariate analysis was carried out using SIMCA-P version 11.0.0.0 Umetrics AB.

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doi:10.1016/j.febslet.2008.07.029

3. Results and discussion

Fig. 1 shows extracted ion traces, taken from an analysis of wild-type (WT) flies, for some metabolites associated with purine catabolism [13]: uric acid, allantoin, xanthine, hypoxanthine, guanine, guanosine and guanosine monophosphate. One hundred and twenty four standards were run in order to establish retention times, some of the standards, e.g. ATP did not elute from the Hilic column probably due to strong anion exchange interactions. Table 1 shows the elemental compositions for compounds related to pathways upstream from uric acid with an indication of the closeness of the mass match to the predicted composition. Uric acid, xanthine, guanine and guanosine all elute at similar retention times but are, importantly, removed from the phospholipid peaks which can cause ion-suppression. Adenosine monophosphate elutes much later than the rest of the metabolites because of its stronger interaction with the hydrophilic layer associated with the column surface. In the current method it was not possible to reliably observe di- and triphosphates; the exception was NAD (Fig. 1).

To carry out chemometric analysis we had to dispense with the chromatographic dimension and sum the ions across the whole chromatographic range. A VBA sub routine was developed to look up the most common mass forms (M + H, M + Na and M + 2/2) of 59 metabolites (Supplementary infor-

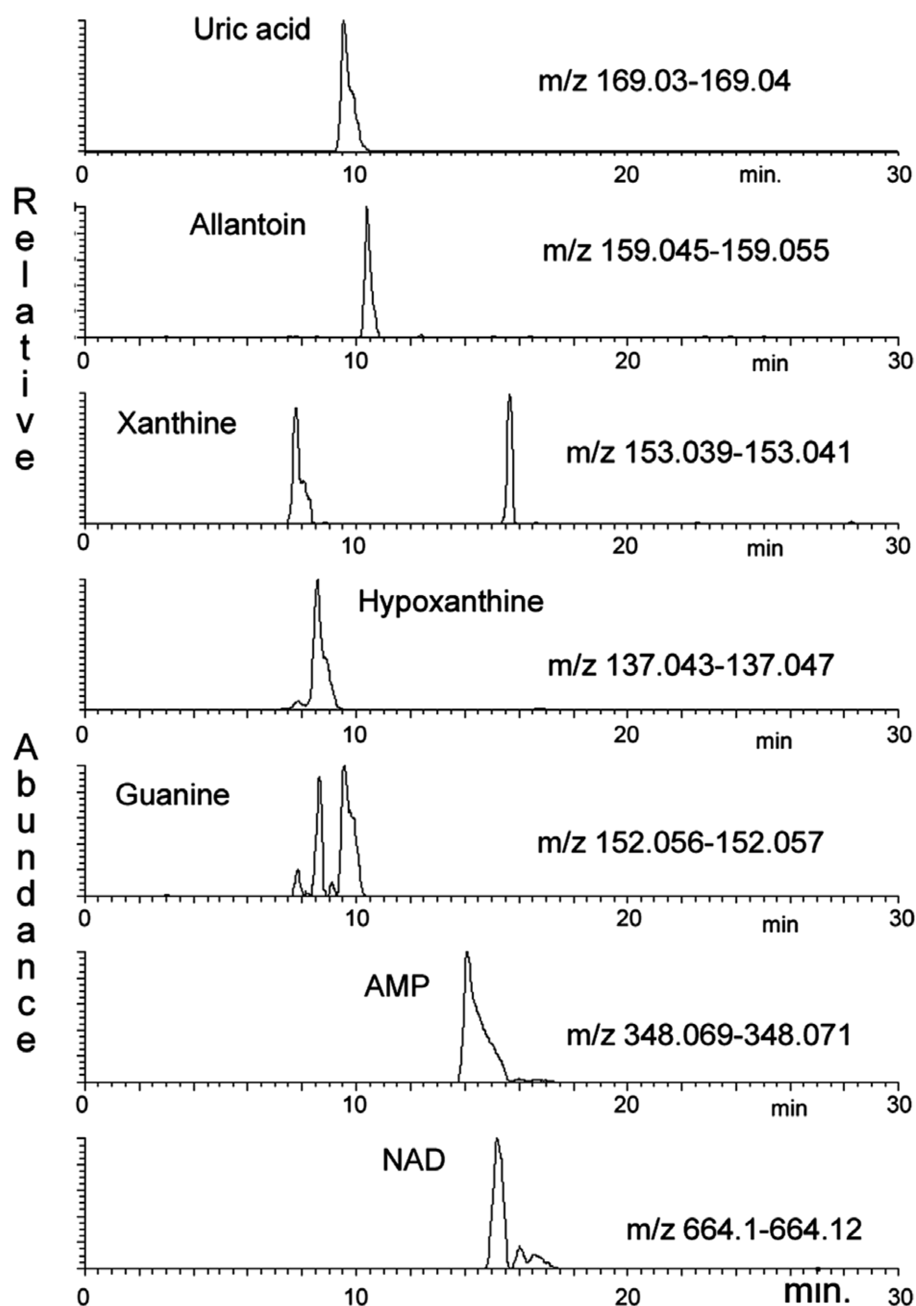

Fig. 1. Narrow-range extracted ion traces extracted across a range of 0.02 amu showing the metabolites related to uric acid biosynthesis from female wild-type *Drosophila*.

Table 1
Mass spectrometric data for metabolites in close proximity, according to the Kegg database (Fig. 2), to the xanthine oxidase lesion in *ry*

Compound	Elemental composition MH^+	Retention time (min)	Deviation (ppm)	Nearest match within 2 ppm	Deviation (ppm)
Uric acid	$C_5H_4N_4O_3$	9.5	0.552	–	–
Allantoin	$C_4H_7N_4O_3$	10.4	0.083	–	–
Xanthine	$C_5H_4N_4O_2$	7.8	−0.340	–	–
Hypoxanthine	$C_5H_4N_4O$	8.6	−0.783	–	–
Guanine	$C_5H_5N_5O$	8.6	−0.22	–	–
Guanosine	$C_{10}H_{13}N_5O_5$	9.6	0.298	$C_{11}H_{13}N_6O_2Na$	−0.669
Biopterin isomer	$C_9H_{11}N_5O_3$	7.3	−0.025	$C_{10}H_{11}N_6Na$	−1.179
Biopterin isomer	$C_9H_{11}N_5O_3$	9.4	−0.109	$C_{10}H_{11}N_6Na$	−1.263
Dihydrobiopterin	$C_9H_{13}N_5O_3$	9.2	0.266	$C_{10}H_{11}N_6Na$	−0.878
Inosine	$C_{10}H_{12}N_4O_5$	8.7	−0.088	$C_{11}H_{20}ONNaS_2$	0.802
AMP	$C_{10}H_{14}N_5O_7P$	14.1	0.02	$C_{15}H_{11}O_4N_5Na$	0.128

mation Fig. S1) directly involved in the purine/pyrimidine metabolism pathway [13] (Supplementary information Table S1). The analytical method was able to observe 23 of these metabolites, which were introduced into SIMCA-P+11 for PCA (Supplementary information Fig. S2) in which *ry* and WT flies were clearly separated. Other metabolites found to be highly variable were adenine, AMP, adenosine and deoxyadenosine, which were up-regulated in *ry* mutants; and allantoin, uridine, uracil and thymine, which were down-regulated in *ry* (Tables 2 and 3). Fig. 2 shows the metabolic pathways closely associated with the conversion of xanthine into uric acid [13]. The mass spectrometric profile rapidly confirms what is known from classical work [5,6], although it clearly detects perturbations further from the original lesion than was previ-

Table 2
Metabolite changes identified in female *ry* flies compared to WT

Metabolite/metabolic pathway	Formula	Recorded mass	Retention time (min)	Hits in METLIN (filtered)	Female	
					Ratio ry/WT	*P*-value
Uric acid related						
Uric acid	$C_5H_4N_4O_3$	169.0356	9.6	1	5.65×10^{-4}	0.000762
Allantoin	$C_4H_6N_4O_3$	159.013	10.5	1		0.000119
Hypoxanthine	$C_5H_4N_4O$	137.0458	8.8	1	15.66	0.000192
Xanthine	$C_5H_4N_4O_2$	153.0407	7.9	1	0.047	0.000319
Guanine	$C_5H_5N_5O$	152.0567	8.6	3	2.83	0.001
Guanosine	$C_{10}H_{13}N_5O_5$	284.0989	9.6	1	2.89	0.003
Biopterin	$C_9H_{11}N_5O_3$	238.0935	9.4	5	3.03	4.1×10^{-9}
Dihydrobiopterin	$C_9H_{13}N_5O_3$	240.1091	9.4	3	5.47	1.21×10^{-6}
Dihydropterin	$C_7H_9N_5O_2$	196.0829	7.9	1	0.13	0.013
Inosine	$C_{10}H_{12}N_4O_5$	269.088	8.7	2	4.69	0.005
AMP	$C_{10}H_{14}N_5O_7P$	348.0704	14.1	2	1.46	0.055
Tryptophan pathway metabolites						
Tryptophan	$C_{11}H_{12}N_2O_2$	205.09715	9.9	2	0.53	0.005
Hydroxytryptophan	$C_{11}H_{12}N_2O_3$	221.0921	9.4	1	1.87	0.028
Kynurenine	$C_{10}H_{12}N_2O_3$	209.921	9.5	1	3.35	5.53×10^{-8}
Hydroxykynurenine	$C_{10}H_{12}N_2O_4$	225.0921	10.7	1	0.405	2.5×10^{-7}
Osmolytes						
Choline glycerophosphate	$C_8H_{21}NO_6P$	258.1101	13.9	1	14.57	4×10^{-11}
Glycerophosphoethanolamine	$C_5H_{14}NO_6P$	216.1101	12.7	1	16.16	2×10^{-10}
Choline	$C_5H_{14}NO$	104.107	13.0	1	1.33	0.006
Pyrimidines						
Cytosine	$C_4H_5N_3O$	112.0505	13.5	1	14.31	0.002
Uracil	$C_4H_4N_2O_2$	113.0346	8.0	1	0.921	0.6
Uridine	$C_9H_{12}N_2O_6$	245.0768	8.0	2	0.9	0.617
Arginine pathway metabolites						
Citrulline	$C_6H_{13}N_3O_3$	176.103	13.4	1	0.299	0.004
Ornithine	$C_5H_{12}N_2O_2$	133.0972	17.6	1	0.376	0.147
L-Homocitrulline	$C_7H_{15}N_3O_3$	190.11862	17.8	1	0.011	1.9×10^{-7}
Misc metabolites						
Alanine	$C_3H_7NO_2$	90.055	13.2	2	1.93	8.4×10^{-8}
Glutamic acid	$C_5H_9NO_4$	148.0604	12.2	4	1.75	0.000314
Glutamine	$C_5H_{10}N_2O_3$	147.0764	12.9	5	1.32	0.000566
Threonine	$C_4H_9NO_3$	120.0655	12.5	2	0.526	3.2×10^{-5}
Nicotinamide	$C_6H_6N2_O$	123.0553	7.8	1	1.55	0.00016
Valine	$C_5H_{11}NO_2$	118.08613	12.0	5	1.06	0.494

Table 3
Metabolite changes identified in male *ry* flies compared to WT

Metabolite/metabolic pathway	Formula	Recorded mass	Retention time (min)	Hits in METLIN (filtered)	Male	
					Ratio ry/WT	*P*-value
Uric acid related						
Uric acid	$C_5H_4N_4O_3$	169.0356	9.6	1	2.5×10^{-9}	0.000596
Allantoin	$C_4H_6N_4O_3$	159.013	10.5	1	0.018	0.002
Hypoxanthine	$C_5H_4N_4O$	137.0458	8.8	1	10.844	4.9×10^{-5}
Xanthine	$C_5H_4N_4O_2$	153.0407	7.9	1	20.665	5.5×10^{-7}
Guanine	$C_5H_5N_5O$	152.0567	8.6	3	1.325	0.073
Guanosine	$C_{10}H_{13}N_5O_5$	284.0989	9.6	1	1.119	0.527
Biopterin	$C_9H_{11}N_5O_3$	238.0935	9.4	5	1.576	0.000518
Dihydrobiopterin	$C_9H_{13}N_5O_3$	240.1091	9.4	3	5.188	1.2×10^{-6}
Dihydropterin	$C_7H_9N_5O_2$	196.0829	7.9	1	0.214	0.006
Inosine	$C_{10}H_{12}N_4O_5$	269.088	8.7	2	2.023	0.011
AMP	$C_{10}H_{14}N_5O_7P$	348.0704	14.1	2	1.075	0.43
Tryptophan pathway metabolites						
Tryptophan	$C_{11}H_{12}N_2O_2$	205.09715	9.9	2	0.36	6.4×10^{-7}
Hydroxytryptophan	$C_{11}H_{12}N_2O_3$	221.0921	9.4	1	0.509	0.004
Kynurenine	$C_{10}H_{12}N_2O_3$	209.921	9.5	1	0.64	0.004
Hydroxykynurenine	$C_{10}H_{12}N_2O_4$	225.0921	10.7	1	0.345	7.1×10^{-7}
Osmolytes						
Choline glycerophosphate	$C_8H_{21}NO_6P$	258.1101	13.9	1	9.56	1.8×10^{-11}
Glycerophosphoethanolamine	$C_5H_{14}NO_6P$	216.1101	12.7	1	9.672	8.7×10^{-8}
Choline	$C_5H_{14}NO$	104.107	13.0	1	0.792	0.09
Pyrimidines						
Cytosine	$C_4H_5N_3O$	112.0505	13.5	1	2.571	0.016
Uracil	$C_4H_4N_2O_2$	113.0346	8.0	1	0.402	0.002
Uridine	$C_9H_{12}N_2O_6$	245.0768	8.0	2	0.5	0.209
Arginine pathway metabolite						
Citrulline	$C_6H_{13}N_3O_3$	176.103	13.4	1	0.348	0.042
Ornithine	$C_5H_{12}N_2O_2$	133.0972	17.6	1	0.128	0.036
L-Homocitrulline	$C_7H_{15}N_3O_3$	190.11862	17.8	1	0.00009	0.00031
Misc metabolites						
Alanine	$C_3H_7NO_2$	90.055	13.2	2	1.259	0.075
glutamic acid	$C_5H_9NO_4$	148.0604	12.2	4	0.871	0.094
Glutamine	$C_5H_{10}N_2O_3$	147.0764	12.9	5	1.11	0.233
Threonine	$C_4H_9NO_3$	120.0655	12.5	2	0.578	0.000221
Nicotinamide	$C_6H_6N2_O$	123.0553	7.8	1	0.732	0.081
Valine	$C_5H_{11}NO_2$	118.08613	12.0	5	0.76	0.016

ously possible and identifies effects in the guanosine/guanine arm of the purine metabolism pathway, that had previously not been described.

In a non-targeted approach a VBA subroutine was developed in-house to eliminate artifacts and lock masses and to correct and to align the samples in the *m/z* direction. All observed masses were introduced into SIMCA-P for PCA, which showed a clear clustering of WT compared with *ry* flies with the males and females of WT and *ry* clustering together (Fig. 3). Metabolites which were found to vary significantly *between* ry and WT were confirmed with an allowed deviation of ±1 ppm. Isotopic patterns were additionally used to prune the number of suggested formulae and confirm the elemental composition [14], the elements that were allowed for elemental matching are shown in Table S2 (Supplementary information). The METLIN data base [15] contains over 26000 metabolite structures and provides useful additional confirmation of compound identity.

It was found that 3-hydroxykynurenine levels were lower in *ry* (Tables 2 and 3) and kynurenine was correspondingly elevated, suggesting that the enzyme responsible for 3-hydroxylating kynurenine displays reduced activity. This should have an effect on pigmentation since the ommochrome pigments are formed in *Drosophila* via this pathway. It was observed that levels of riboflavin which is necessary for the biosynthesis of the FAD co-factor required for hydroxylation of kynurenine, were much lower in *ry*. This may be purely due to malabsorption of the vitamin in *ry*.

Another observation is that glycerophosphotidylcholine (GPC) levels are far higher in *ry* (Tables 2 and 3). GPC is a counteracting osmolyte and has been found to accumulate in mammalian renal medullary cells, in response to high extracellular concentrations of NaCl or urea, maintaining osmotic balance without denaturing proteins within the cell which would occur in the presence of high concentrations of inorganic ions [16,17]. *Inebriated* mutants of *Drosophila*, which lack an osmolyte transporter in the malpighian tubule, are hypersensitive to NaCl [18]. Terrestrial insects are considered to be uricotelic eliminating nitrogenous waste as insoluble uric acid crystals, thus conserving water [19]. As *ry* mutants are unable to make uric acid, elimination is likely to occur in the form of hypoxanthine and xanthine. Although some xanthine is deposited as

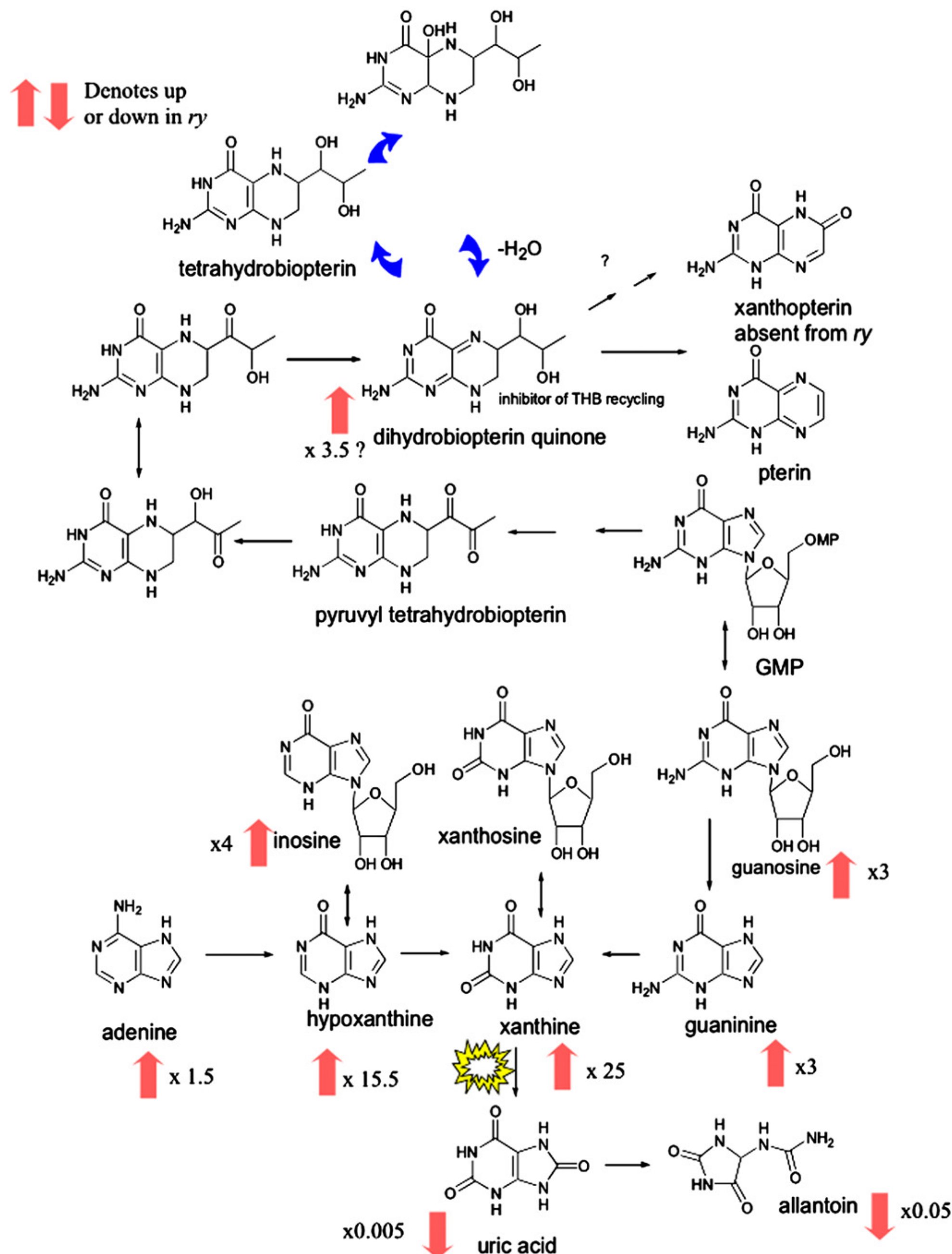

Fig. 2. Some of the metabolic network in close proximity to the ry lesion.

renal calculi in *ry* mutants [5], both xanthine and particularly hypoxanthine are more water soluble than uric acid [20] thus nitrogen excretion in *ry* may require higher rates of water loss. Accumulation of GPC in tissues may thus help to compensate for osmotic stress.

Arginine metabolism is also affected in *ry*. Perhaps the observed accumulation of dihydrobiopterin (DHBT) causes an inhibition of tetrahydrobiopterin (THBT) recycling (Fig. 2). One of the isomers of DHBT is known to be an inhibitor of THBT regeneration [21] and THBT is required as a co-factor in arginine oxidation which results in the formation of NO and citrulline. Accumulation of DHBT in vascular tissue may have a role in insulin resistance where it inhibits vasodilation through inhibiting NO production [22].

Tables 2 and 3 list some additional metabolites that are significantly altered in *ry* mutants including pyrimidine metabolites, such as cytosine and uracil. *Ry* has been widely used as a selectable marker in mutant stocks, and on early synthetic P-element constructs [23]. It is clear that the impact of *ry* on the fly ramifies widely; and so *ry*, like ebony, is a particularly poor choice of marker because of its pleiotropy.

Since a third of genes in a typical metazoan genome have neither obvious functions based on their encoded protein structures nor evocative tissue distributions, it has been considered that reverse genetics, coupled to painstaking analysis of informative functional phenotypes, is the best approach to identify function in both plants and animals [24,25]. However, such studies are critically limited by the lack of informative

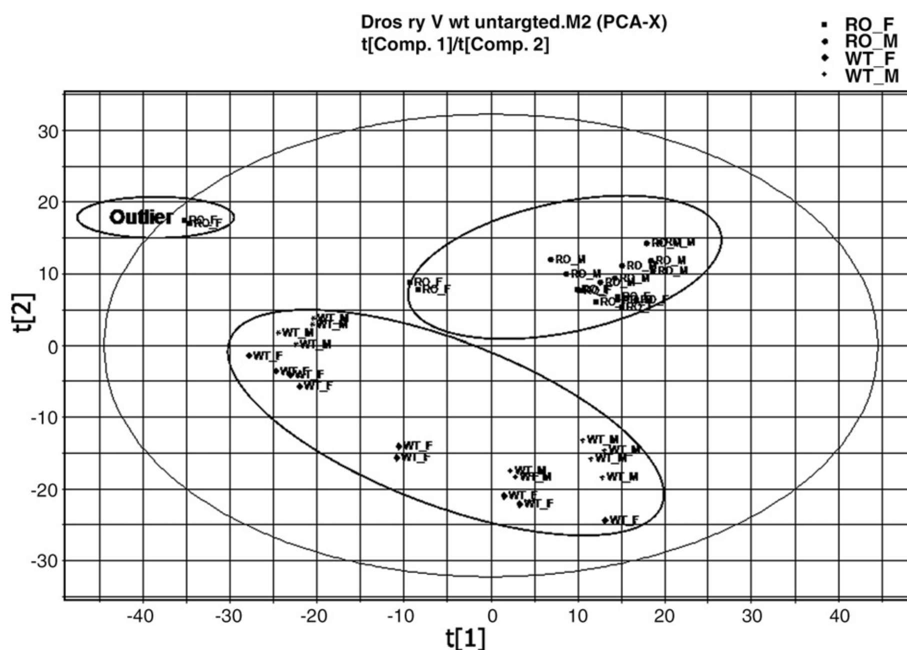

Fig. 3. PCA separation of male and female WT flies from male and female *ry* flies based on global metabolite profile.

phenotypes to study in genetic model organisms – the so-called phenotype gap [2,26] – metabolomics can provide an alternative method of adding value to the many thousands of unstudied mutant stocks available today. Metabolomics has already been employed in pioneering studies in *Drosophila* [27–29]. The combination of *Drosophila* and ZICHILIC-FTMS, with the downstream computational methods we describe here, can provide precision and comprehensiveness that will allow rapid progress.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2008.07.029.

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