Supporting information files

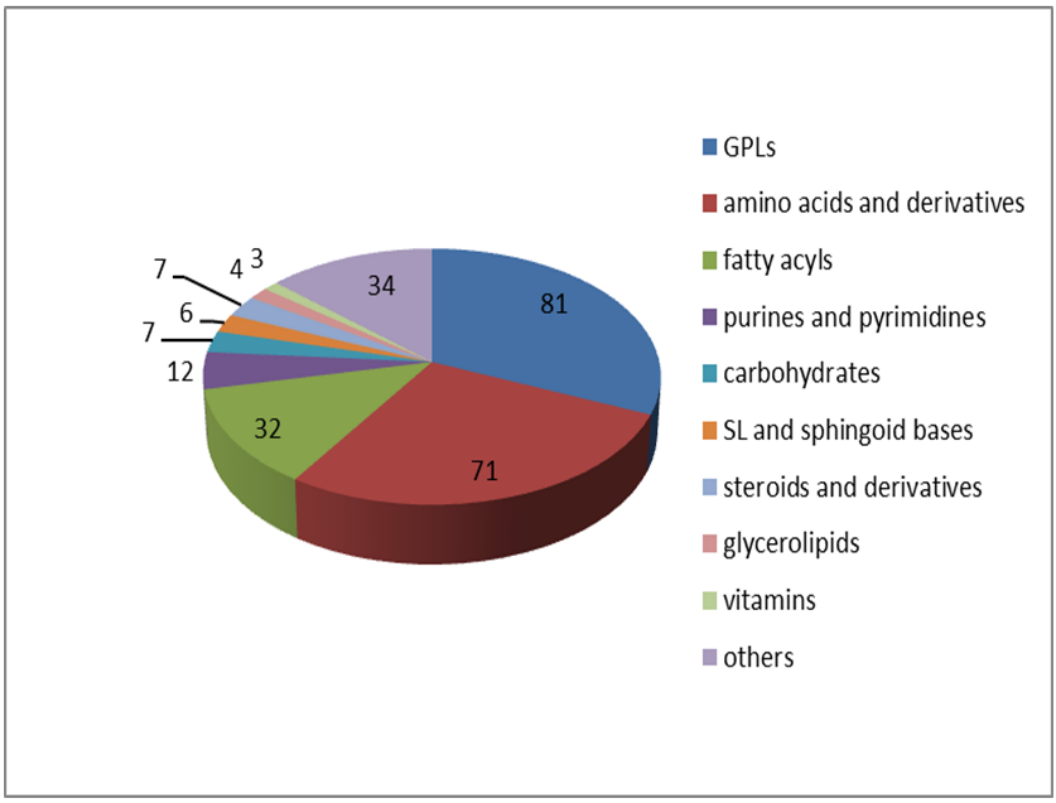
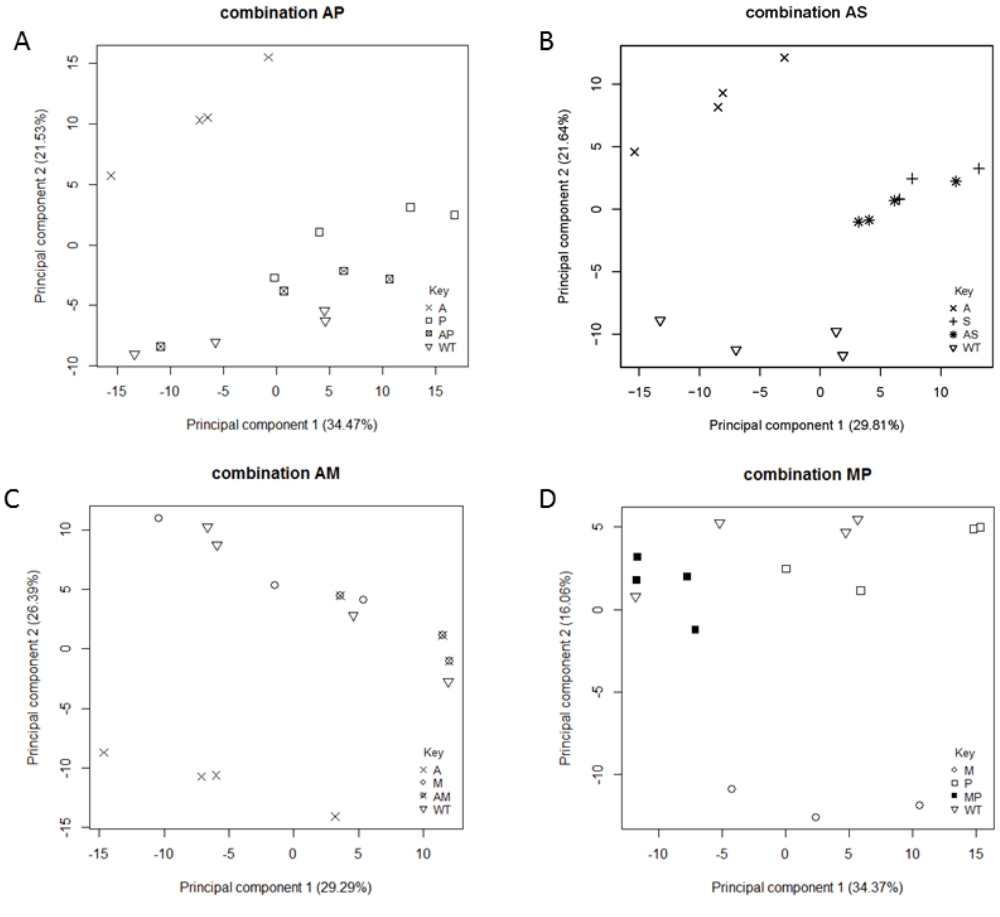
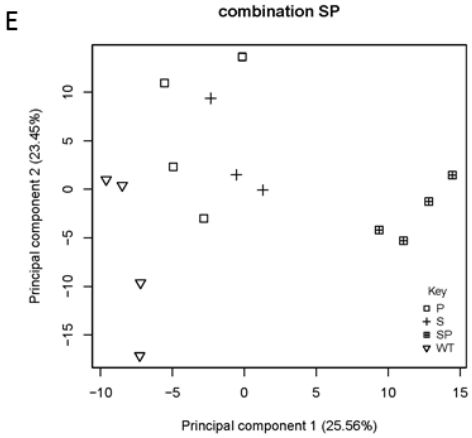


Figure S1: Quantitative distribution of 257 putatively identified metabolites in metabolic classes. The 257 metabolites belonged to the following classes (in order of quantitative representation) and are represented in a piechart format: glycerophospholipids (GPLs), amino acids and derivatives, fatty acyls, purines and pyrimidines, carbohydrates, sphingolipids (SL) and sphingoid bases, steroids and derivatives, glycerolipids and vitamins and cofactors.



9



10

11 **Figure S2: Principal component analysis of CTR *L. donovani* promastigote lines (AP, AS, AM,**
12 **MP and SP) and their respective single-resistant lines.** Principal component analysis (PCA)
13 of AP (plot A), AS (plot B), AM (plot C), MP (plot D) and SP (plot E) CTR lines and their
14 respective single-R lines (A, P, S, M) based on the quantitative measurements of all 257

putatively identified compounds. In this study three biological replicates were removed due to signal intensity drift: M_BR1, S_BR2 and AM_BR3. WT represents WTM.

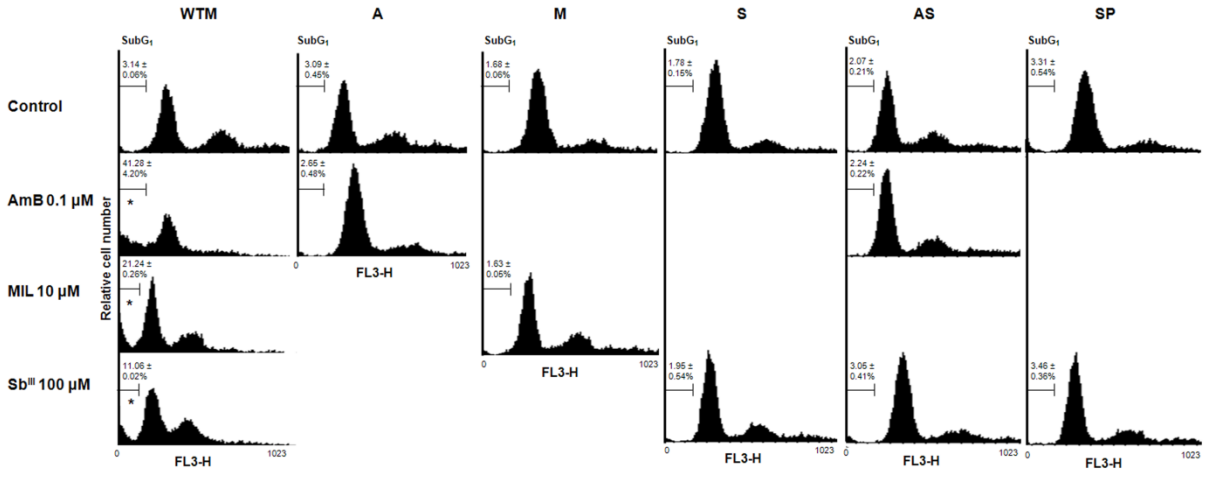


Figure S3. DNA content analysis in *L. donovani* lines. Control (WTM), A, M, S, AS, and SP **.** *L. donovani* lines, were left untreated or exposed to 0.1 µM AmB, 10 µM MIL, or 100 µM Sb^{III} for 48 h. Promastigotes were incubated with 1 µg/ml PI for 1 h in the dark at room temperature. The distribution of DNA content was analyzed by flow cytometry. The percentages of cells in the $subG_1$ phase, expressed as means ± SD for three independent experiments were significantly different from control (untreated) values by Student's *t*-test (*: $p< 0.01$).

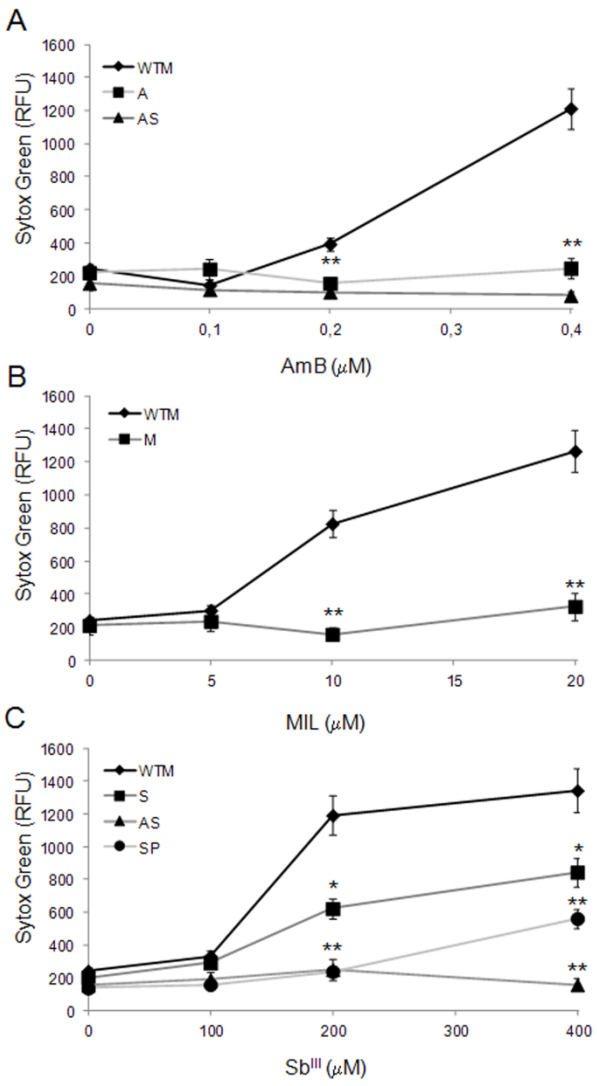


Figure S4. Cell membrane integrity in *L. donovani* lines. Control (WTM), A, M, S, AS and SP lines were left untreated or exposed to increasing concentrations of (A) AmB, (B) MIL, or (C) Sb^{III} for 48 h. Parasites were incubated with 2 µM Sytox Green for 10 min at 28°C. The fluorescence intensity was determined by flow cytometry analysis and expressed as Relative Fluorescence Units (RFU). Data are the means ± SD of three independent experiments. Significant differences versus WTM line were determined by Student's *t*-test (*: $p< 0.005$; **: $p< 0.001$).

Table S1: Settings of triple quadrupole tandem-mass spectrometer Xevo TQS (Waters Corp) for MS detection of Amphotericin B (AmB)

Source (ESI+) and analyzer	Settings
Capillary voltage (kV)	3.30
Cone voltage (V)	138.28
Source temperature (°C)	150
Desolvation temperature (°C)	650
Cone gas flow (L/h)	150
Desolvation gas flow (L/h)	1000
Collision gas flow (mL/min)	0.15

Table S2: Overview of CTR-collective (shared by AS and SP) and CTR specific metabolic changes (only in AS or only in SP). Metabolite changes were considered to be biologically significant when the ratio of signal intensity between the resistant line and the WTM line (fold change) was higher than 2 (significant increase) or lower than 0.5 (significant decrease) and statistically significant ($p < 0.05$ and $p < q$); *AdoMet (*S*-adenosylmethionine) was found to be increased significantly in both AS and SP lines but not by a 2-fold in AS (fold change: 1.78). **Mevalonate was found to be decreased significantly in both AS and SP lines but not by a 0.5-fold in AS (fold change: 0.53). Tryptophan (Trp) was found to be increased a 2.58-fold in the AS line with $p = 0.0146$ but $p > q$. sterol A: a secosteroid with monoisotopic mass 398.318484 Da, sterol B: a secosteroid with monoisotopic mass 412.333833 (an ergostatetraene-diol and its isomers). *N*-Ac-Phe: N-acetylphenylalanine; sedoheptulose-7-P: sedopheptulose-7-phosphate.

Metabolic pathways	Δmb		
	shared by AS and SP (13 increases, 3 decreases)	**only detected in AS** (13 increases, 0 decreases)	**only detected in SP** (14 increases, 11 decreases
proline biosynthesis	x (proline, (iso)leucyl-proline, pyrroline)	x (pyrroline carboxylate)	x (proline betaine)
trans-sulfuration pathway	x (pyridoxine, AdoMet*)	x (methionine)	
aromatic amino acids	x (N-Ac-Phe)	X (Trp***)	
lipid metabolism	x (glyceric acid, glycerol-3-phosphate and 2 fatty acyls, copaene, dehydrosphinganine, mevalonate**)	x (fatty acyls: 1 increased, 1 decreased)	x (GPLs: 2 unsaturated, 3 low saturated, 5 high unsaturated, 1 low unsaturated)
tryptophan degradation pathway		x (indole acrylate, Trp***)	
acylglycines		x (caproylglycine)	x (valerylglycine)
others	x (erythrulose, hexitol, hypoxanthine, glutamylalanine, adenosine)	x (pentose ring, ovothiol, sugar phosphate, Asp-Arg, sterol A, gamma-glutamyl-gamma-aminobutyraldehyde, 2-C-Methyl-D-erythritol 4-phosphate)	x (guanidinobutanoic acid, sedoheptulose-7-P, acetylhomoserine, pentadecanal, porphobilinogen peptides (4), sterol B

Table S3: Differential metabolites detected in A, M, S, P, AS and SP compared to WTM. Major representatives are shown for each metabolic group. Underlined metabolites were detected in their respective CTR lines (mentioned in brackets which line it concerns: AS or SP). More detailed information on specific metabolites can be found in the supplementary Table S4. PPP: pentose phosphate pathway; *N*-Ac-Phe: *N*-acetyl-phenylalanine; *N*-Ac-His: *N*-acetyl-histidine, AdoMet: *S*-adenosylmethionine

Differential metabolites	A	M	S	P	AS	SP
Significant increases (> 2 fold)	n=17 GPL: 6 low unsaturated amino acids and derivatives: <u>*N*-acetyl-Phe</u> (AS), tyrosine, *N*-Ac-His, dimethylarginine	n=19 GPL: 1 low unsaturated, 1 saturated, 3 high unsaturated fatty acyls (3) amino acids and derivatives: <u>proline</u> (SP),	n=25 <u>fatty acyls</u> (6) (4 shared with AS, 3 with SP) GPL and building blocks: <u>3 saturated GPLs</u> (2 shared with	n=12 GPL : 1 low unsaturated <u>fatty acyls (2)</u> (SP) amino acids and derivatives: <u>proline</u> (SP), <u>(iso)leucylproline</u> (SP),	n=28 amino acids and derivatives: (iso)leucylproline, proline, pyrroline, pyrroline carboxylate, *N*-Ac-Phe, methionine,	n=30 GPL and building blocks: 2 saturated, 5 high unsaturated, 3 low unsaturated, glycerol-3-phosphate PPP:sedoheptulose-7-P fatty acyl (4)

	others: pyridoxine (AS), uracil (AS), hypoxanthine (AS), caproylglycine (AS), 3-hydroxy-9,10-seco-cholestatriene-one (AS), erythrulose	(iso)leucylproline (SP), pyrroline (SP) others: hypoxanthine (SP), pyridoxine (SP), erythrulose, pentose ring, indole acrylate	SP), glyceric acid (AS,SP), PPP: pentose ring (AS), sedoheptulose-7-P (SP) Amino acids and derivatives: proline (AS,SP), (iso)leucyl-proline (AS,SP), pyrroline (AS,SP), methionine (AS), *N*-Ac-Phe (SP), AdoMet (SP), glutamylalanine (AS,SP)leucine/isoleucine, Others: pyridoxine (AS,SP), caproylglycine (AS), cysteinylglycine disulfide	pyrroline (SP), 4-methylene-glutamine, others: hypoxanthine (SP), pyridoxine (SP), uracil	glutamylalanine, fatty acyls (4) others: hypoxanthine, pyridoxine, pentose ring, indole acrylate, hexitol, glycerol-3-phosphate, uracil, 1 steroid, ovothiol, caproylglycine	amino acids and derivatives: proline, pyrroline, (iso)leucylproline, proline betaine, AdoMet, Glu-Ala, *N*-Ac-Phe, guanidinobutanoic acid others: hexitol, hypoxanthine
Significant decreases (< 0.5 fold)	n=15 copaene (AS) GPL: 6 high unsaturated, fatty acyls (4), others: C16 sphinganine, porphobilinogen, mevalonate	n=1 fatty acyl	n=4 adenosine (AS, SP), mevalonate (SP), 1 sterol (SP), 1 fatty acyl	n=2 mevalonate (SP), fatty acyl (1)	n=3 adenosine, dehydroalanine, 3-copaene	n=18 1 GPL: low unsaturated 2 fatty acyls amino acids and derivatives: dehydrolalanine, 3 peptides others: porphobilinogen, Ac-homoserine, valerylglycine, adenosine, 2 steroids, copaene,mevalonate

Table S4 (Excel file): **List of 257 unique biological analytes.** List of 257 putatively identified metabolites with for each compound the following information: (A) detected mass; (B) ppm deviation between detected mass and theoretical mass of assumed metabolite identification; (C) chromatographic retention time; (D) converted chromatographic retention time; (E) putative metabolite identification; (F) compound class; (G) compound subclass; (H-

AV) signal intensity in each sample (each line is color-coded); (AW-CC) ratio of average signal intensity of resistant line versus WTM line followed by *p* value of a t-test assuming unequal variance rank and *q* value (Benjamini-Hochberg); (CK) dilution p value; (CL) dilution Pearson's correlation coefficient; (CM) database in which the metabolite was detected. Ratios in bright red were metabolites with a fold change higher than 2 and with $p < 0.05$ and $p < q$, rations in light red were metabolites with a fold change higher than 2 but with $p > 0.05$ or $p > q$ ratios. Ratios in dark blue were metabolites with a fold change lower than -2 with $p < 0.05$ and $p < q$, ratios in light blue were metabolites with a fold change lower than -2 but with $p > 0.05$ or $p > q$ ratios. Ratios in bold had a significant corresponding p value (t-test assuming unequal variance) and $p < q$. BR: biological replicate; KEGG: Kyoto Encyclopedia of Genes and Genomes; NA: not detected. Glycerophospholipids (GPLs) have been divided into three classes, abbreviations should be interpreted as follows: GPL(x:y/z), where x represents the number of carbons in the fatty acid side chain(s), y represents the number of double bonds, and z represents the number of side chains. A distinction is made for saturated GPLs (no double bonds, y = 0), low unsaturated GPLs (y = 1 or 2) and high unsaturated GPLs (y > 2) (1).

Detailed information on significant metabolic changes in the single resistant and CTR lines

In the AmB resistant line (A line) most changes were located in the glycerophospholipid class (Table S2). Twelve GPLs were significantly changed: 6 low unsaturated GPLs were increased and 6 high unsaturated GPLs were decreased. No changes were detected in ergosterol (the target of AmB), but the sterol with monoisotopic mass of 398.318484 Da was found to be a 2-fold increased. The levels of all aromatic amino acids (Tyr, Phe, Trp) and the derivative *N*-acetylphenylalanine were increased. Other increased metabolites were uric acid

89 and hypoxanthine (both purine bases), dimethylarginine, *N*-acetylhistidine and
90 caproylglycine (Table S2). Decreases were i.a. detected in the fatty acyls (4), mevalonate and
91 sphingolipids (1).
92
93 **In the MIL resistant line (M line)** 19 out of 20 significantly differential metabolites were
94 increased (Fig. 1). Five GPLs were found of which 4 GPCs with an ether alkyl chain, three of
95 them were connected in the ether phospholipid biosynthesis pathway (GPC(O-34:2/2);
96 GPC(O-34:2/3); GPC(O-34:2/4)). The amino acids (and derivatives) dimethylarginine (a
97 natural metabolic by-product of arginine), proline and (iso)leucylproline, 3 fatty acyls, indole
98 acrylate and hypoxanthine were also found to be increased (Table S2). A particular lipid with
99 a mass of 453.3219 was found in all M resistant lines (M, AM, MP) but was not detected in
100 the other lines. A similar finding was reported in *L. infantum* by (2). This mass either
101 correlated with a GPC (O-14:0/1) or a formate adduct (CH_2O_2) of MIL. A serial dilution of
102 different batches of MIL from different suppliers on LC-MS showed this mass to be present
103 in every batch, confirming the hypothesis that this lipid is not parasite-derived but a MS
104 artefact.
105
106 **In the Sb^{III} resistant line (S line)**, 25 metabolites showed to be significantly increased. Of
107 these, the fatty acyls (7) showed the most pronounced fold changes (between 2.2 and 21.8)
108 compared to the WTM. Two metabolites (pentose-ring and sedoheptulose-7-phosphate)
109 belong to the pentose phosphate pathway, which generates NADPH; a cofactor crucial to
110 reduce oxidized metabolites such as glutathione disulfide. Other interesting increased
111 metabolites were cysteinylglycine disulfide (8-fold, glutathione metabolism), the amino acids
112 proline (7-fold increase), methionine and Leu/Ile, *N*-acetylphenylalanine, S-

adenosylmethione (cysteine trans-sulfuration pathway) and caproylglycine (an acylglycine) (Table S2).

In the PMM resistant line (P line), fewer metabolic changes were detected (14) and were scattered in different metabolic classes (Table S2). Increased metabolites concern fatty acyls (2), a low unsaturated GPC, proline, pyrroline and (iso)leucylproline (all belonging to the proline metabolic pathway), hypoxanthine, uracil (2 nucleobases), erythrulose and pyridoxine (vit B6 metabolism).

In the AS line, a similar number of differential metabolites (31) was observed compared to the respective single-R lines (S: 32; A: 29) (Fig. 1). However, only 3 differential metabolites were shared by the three lines, i.e. caproylglycine, pyridoxine and *N*-acetylphenylalanine (Table 1). The levels of indole acrylate and tryptophan were increased a 2-fold in all three lines but not in a significant matter ($p < 0.05$ but $p > q$). S-adenosylmethionine (AdoMet) and methionine were also significantly increased in both the S and AS line (cysteine trans-sulfuration pathway). Ovothiol and hexitol were only found to be increased in the AS line.

Of its 31 increased metabolites**, the SP line** shared 6 increased metabolites with the S and P line related to the proline metabolism, pyridoxine and two fatty acyls. With the S line, it shared 7 metabolites located in different pathways (GPL, amino acids, PPP) and with the P line only 2 metabolites that however also showed a non-significant increase in the S line (erythrulose, hypoxanthine). Metabolites that were only increased in the SP line are mainly unsatured GPLs (8) and the building block glycerol-3-phosphate. Of the 18 decreased metabolites, only 1 was shared with both single-R lines (mevalonate) and 2 with S (sterol,

adenosine). Other decreased metabolites were scattered in different metabolic classes (amino acids, fatty acyls, sphingolipids) (Table S2).

In the AM line only 3 significant increases were detected: GPC-(O-14:0/1), glutamylalanine and hypoxanthine. The latter is shared with both A and M single-R lines and the ether GPL is shared only with the M line.

In the AP line only 4 significant increases were detected: GPC(O-34:2/2), erythrulose, uracil and (iso)-leucyl-proline. All these metabolites (except for the latter one which was not increased in the A line) were shared with the single-R lines A and P. Likewise, a decrease for mevalonate was observed in the AP, A and P lines.

In the MP line, 7 metabolites were found to be increased of which 3 belonged to the proline metabolism and were shared with both single-R lines. The 4 other differential metabolites (decreases) belonged to the lipid metabolism and only one was shared with the M line (GPC-(O-14:0/1)).

153 Reference List

154

155 1. **Berg M, Vanaerschot M, Jankevics A, Cuypers B, Maes I, Mukherjee S, Khanal B, Rijal S, Roy S,**
156 **Opperdoes F, Breitling R, Dujardin JC.** 2013. Metabolic adaptations of Leishmania donovani in
157 relation to differentiation, drug resistance, and drug pressure. Mol Microbiol. **90**:428-442.

158 2. **Vincent IM, Weidt S, Rivas L, Burgess K, Smith TK, Ouellette M.** 2014. Untargeted
159 metabolomic analysis of miltefosine action in Leishmania infantum reveals changes to the
160 internal lipid metabolism. Int J Parasitol Drugs Drug Resist. **4**:20-27.
161
162