**Supporting Information**

**An environmentally relevant mixture of polychlorinated biphenyls (PCBs) and polybrominated diphenylethers (PBDEs) disrupts mitochondrial function, lipid metabolism and neurotransmission in the brain of exposed zebrafish and their unexposed F2 offspring.**

Mélanie Blanc1\*, Sébastien Alfonso2,3, Marie-Laure Bégout2, Célia Barrachina4, Tuulia Hyötyläinen1, Steffen H. Keiter1, and Xavier Cousin2,5

1Man-Technology-Environment Research Centre (MTM), School of Science and Technology, Örebro University, Fakultetsgatan 1, S-701 82 Örebro, Sweden

2MARBEC, Univ. Montpellier, CNRS, Ifremer, IRD, Route de Maguelone, F-34250, Palavas-les-Flots, France

3COISPA Tecnologia & Ricerca, Stazione Sperimentale per lo Studio delle Risorse del Mare, Via dei Trulli, n 18 70126 Bari, Italy

4MGX, Univ. Montpellier, CNRS, INSERM, Université Montpellier 2, Place Eugène Bataillon, F- 34095, Montpellier, France

5Université Paris-Saclay, AgroParisTech, INRAE, GABI, Domaine de Vilvert, F-78350 Jouy-en-Josas, France

\*Corresponding author: mblanc.uni@gmail.com

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# **Section S1:** Chemical characterization of the fish diets and experimental design.

## **Table S1**:Composition of MIX and Control diets.List of PCB and PBDE congeners along with the chlorine and bromine numbers. Targeted and measured concentrations (ng g-1 ww) in MIX (n =12) diet are indicated (mean±SE), as well as the spiking efficiency. For the Control diet, the concentration of each congener as well as the number of samples (n = 16) in which the congener was detected are indicated (Occurrence column). n.d.: not detected. LOD: limit of detection.

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  |  | MIX diet |  |  |  |  | Control diet |  |  |
| Congener |  |  |  |  |  |  |  |  |  |  |  |
|  | Br/Cl | Targeted concentration (ng g-1) | Measured concentration (ng g-1) | Spiking efficiency (%) | Measured concentration (ng g-1) | Occurrence (n) |
| CB-8 | 2 | 28 | 27.20 | ± | 1.21 | 97.1 |  | 0.20 | ± | 0.05 | 3 |
| CB-18 | 3 | 38 | 32.70 | ± | 1.37 | 86.1 |  | 0.60 | ± | 0.28 | 4 |
| CB-28 | 3 | 75 | 69.00 | ± | 3.35 | 92 |  | 0.30 | ± | 0.07 | 5 |
| CB-31 | 3 | 42 | 47.40 | ± | 1.74 | 112.9 |  | 0.30 | ± | 0.05 | 4 |
| CB-44 | 4 | 65 | 66.50 | ± | 1.83 | 102.3 |  | 0.40 | ± | 0.23 | 4 |
| CB-49 | 4 | 54 | 53.20 | ± | 4.67 | 98.5 |  | n.d. |  | - | n.d. |
| CB-52 | 4 | 65 | 64.10 | ± | 2.76 | 98.6 |  | 0.20 | ± | 0.04 | 6 |
| CB-77 | 4 | 30 | 31.80 | ± | 1.54 | 106 |  | n.d. |  | - | n.d. |
| CB-101 | 5 | 150 | 151.90 | ± | 6.47 | 101.3 |  | 0.90 | ± | 0.48 | 8 |
| CB-105 | 5 | 76 | 78.90 | ± | 5.43 | 103.8 |  | 0.40 | ± | 0.18 | 8 |
| CB-110 | 5 | 166 | 170.60 | ± | 7.03 | 102.8 |  | 0.60 | ± | 0.19 | 8 |
| CB-118 | 5 | 110 | 107.30 | ± | 3.05 | 97.5 |  | 0.60 | ± | 0.16 | 8 |
| CB-128 | 6 | 37 | 32.60 | ± | 1.30 | 88.1 |  | 0.20 | ± | 0.07 | 6 |
| CB-132 | 6 | 71 | 68.70 | ± | 6.46 | 96.8 |  | 0.20 | ± | 0.11 | 5 |
| CB-138 | 6 | 207 | 199.60 | ± | 4.94 | 96.4 |  | 0.60 | ± | 0.30 | 8 |
| CB-149 | 6 | 158 | 161.70 | ± | 2.47 | 102.3 |  | 0.80 | ± | 0.24 | 8 |
| CB-153 | 6 | 280 | 270.40 | ± | 14.24 | 96.6 |  | 0.60 | ± | 0.42 | 8 |
| CB-156 | 6 | 38 | 34.10 | ± | 3.71 | 89.7 |  | 0.10 | ± | 0.01 | 7 |
| CB-170 | 7 | 71 | 69.10 | ± | 4.76 | 97.3 |  | <LOD |  | - | 8 |
| CB-180 | 7 | 130 | 129.30 | ± | 5.55 | 99.4 |  | 0.30 | ± | 0.12 | 8 |
| CB-187 | 7 | 35 | 32.60 | ± | 2.91 | 93.1 |  | 0.60 | ± | 0.45 | 4 |
| CB-194 | 8 | 35 | 33.60 | ± | 3.56 | 96 |  | <LOD |  | - | 4 |
| Sum PCBs |  | 1991 | 1932.30 | ± | 90.35 | 97.05 |  | 7.90 |  | 3.45 |  |
| BDE-28 | 3 | 9.95 | 10.78 | ± | 0.60 | 108.3 |  | 0.03 | ± | 0.01 | 5 |
| BDE-47 | 4 | 49.97 | 49.93 | ± | 2.91 | 99.9 |  | 0.35 | ± | 0.13 | 16 |
| BDE-100 | 5 | 14.94 | 15.95 | ± | 1.19 | 106.8 |  | 0.09 | ± | 0.04 | 16 |
| BDE-99 | 5 | 30.25 | 30.25 | ± | 1.63 | 100.0 |  | 0.11 | ± | 0.07 | 16 |
| BDE-153 | 6 | 9.74 | 10.18 | ± | 0.56 | 104.5 |  | 0.03 | ± | 0.004 | 6 |
| BDE-183 | 7 | 100.48 | 130.20 | ± | 12.65 | 129.6 |  | 0.14 |  | - | 1 |
| BDE-209 | 10 | 195.73 | 232.53 | ± | 31.27 | 118.8 |  | 0.17 | ± | 0.11 | 16 |
| Sum PBDEs |  | 411.1 | 479.82 | ± | 50.81 | 116.7 |  | 0.92 | ± | 0.36 |  |



**Figure S1**: Experimental procedure for exposure and sampling. S: novel tank diving test and sampling time for lipidomic and transcriptomic analyses; R: reproduction to produce the next generation.

# **Section S2**: Lipidomic analyses

The UHPLC system used in this work was a 1290 Infinity system from Agilent Technologies (Santa Clara, USA). The system was equipped with a multi sampler (maintained at 10 °C), a quaternary solvent manager and a column thermostat (maintained at 50 °C). Separations were performed on an ACQUITY UPLC® BEH C18 column (2.1 mm × 100 mm, particle size 1.7 µm) by Waters. The mass spectrometer coupled to the UHPLC was a 6545 quadrupole time of flight (Q-TOF) from Agilent Technologies interfaced with a dual jet stream electrospray (dual ESI) ion source. All analyses were performed in positive ion mode and MassHunter B.06.01 (Agilent Technologies) was used for all data acquisition. MS data processing was performed using the open source software MZmine 2.34. The following steps were applied in the processing: 1) Mass detection with a noise level of 750 2) Chromatogram builder with a min time span of 0.08 min, min height of 1000 and a m/z tolerance of 0.006 m/z or 10.0 ppm, 3) Chromatogram deconvolution using the local minimum search algorithm with a 70% chromatographic threshold, 0.05 min minimum RT range, 5% minimum relative height, 1000 minimum absolute height, and a peak duration range of 0.08 - 2.0, 4) Isotopic peak grouper with a m/z tolerance of 5.0 ppm, RT tolerance of 0.05 min, maximum charge of 2 and with the most intense isotope set as the representative isotope, 5) Join aligner with a m/z tolerance of 0.006 009 or 10.0 ppm and a weight for of 2, a RT tolerance of 0.1 min and a weight of 1 and with no requirement of charge state or ID and no comparison of isotope pattern, 6) Peak list row filter with a minimum of 10% of the sample), 7) Gap filling using the same RT and m/z range gap filler algorithm with an m/z tolerance of 0.006 009 m/z or 1011.0 ppm, 7) Identification of lipids using a custom database search with an m/z tolerance of 0.006 m/z or 10.0 ppm and a RT tolerance of 0.1 min, 8) Filtering of adducts < 50% of abundance of the molecular peak with a RT tolerance of 0.05 min and 0.006 m/z or 6.0 ppm, 9)Normalization using ISTDs for identified lipids and closest ISTD for unknown lipids. Concentrations were calculated using lipid-class specific calibration curves. Identification was based on in-house library, constructed with MS/MS data and retention times. Most of the lipids were detected and identified as [MH+], except for di-and triacylglycerol, which were detected as ammonium adducts, and cholesteryl esters which were detected as their cholesteryl fragment ion. Quality control was performed throughout the dataset by including blanks, pure standard samples, extracted standard samples and control plasma samples. Relative standard deviations (%RSDs) for internal standards in all samples was on average 14.4% (raw variation) and the %RSD for lipid concentrations in the pooled samples (n = 8) was on average 24%. The distribution of samples and QC can be visualized in Figure S2. A detailed list of individual feature concentrations obtained per sample is available in the Excel file.

## **Table S2**: list of abbreviations used for biological groups of lipids

|  |  |
| --- | --- |
| Biological group | Abbreviation |
| Phospholipids | PL |
| Triacylglycerols | TG |
| Diacylglycerols | DG |
| Monoacylglycerols | MG |
| Sphingomyelines | SM |
| Phosphatidic acids | PA |
| Lysophosphatidylethanolamines | lysoPE |
| Lysophosphatidylcholines | lysoPC |
| Phosphatidylethanolamines | PE |
| Phosphatidylcholines | PC |
| Cholesteryl esters | CE |
| Ceramides | Cer |



## **Figure S2**: PCA plot showing the distribution of all lipidomic brain samples and QC pooled samples.



## **Figure S3:** Heatmap displaying the changes in lipid profiles in brain of female fish exposed to MIX. Results are shown as log2(fold-change) (MIX/Control) for lipid classes (rows). Rows indicate lipid classes: triacylglycerols (TG), diacylglycerols (DG), monoacylglycerols (MG), all phospholipids (PL), phosphatidic acids (PA), phosphatidylserine (PS), phosphatidylethanolamines (PE), lysophosphatidylethanolamines (lysoPE), phosphatidylcholines (PC), lysophosphatidylcholines (lysoPC), phosphatidylinositols (PI), sphingomyelines (SM), Ceramides (Cer), and cholesteryl esters (CE). Columns indicate F0 (F0\_F) and F2 females (F2\_F). Symbols indicate statistical significance with #: p-value<0.05 (n=4).

# **Section S3**: quantitative Polymerase Chain Reaction (qPCR) validation

Biological validation of the RNA-Sequencing results using qPCR was performed using RNAs from replicate individuals which did not underwent RNA-Sequencing (6 per treatment). RNAs were extracted using TriPrep extraction kit and following kit guidelines (Macherey-Nagel). They were quantified with a Biodrop μLITE (BioDrop, UK) and cDNAs were synthetized from 500 ng of RNA template. Final samples were diluted 10 times prior to use for qPCR. Primers (Eurofins Scientific, Luxembourg) were designed using the online free software Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) (Table S3). Specificity, efficiency, and linearity ranges were established for all primer pairs using melting and standard curve analyses. Each qPCR reaction was processed using 2X SYBR® FAST MasterMix (KAPA Biosystems, France), 200 nM of each primer, 2 μl of cDNA dilution; topped up to 12 μl with MilliQ water. Reactions were run in technical duplicates. Presence of gDNA contamination was controlled by running samples that did not undergo reverse transcription. Results were normalized to the expression of *rpl13a* and *b2m* as they showed the highest stability among 4 investigated genes (i.e. *eef1a1*, *b2m*, *actb1* and *rpl13a*) (data not shown). Fold-changes were calculated according to Pfaffl method using REST software [3].

## **Table S3**: Table of primers used in the study.

|  |  |  |  |
| --- | --- | --- | --- |
| Gene | Forward primer | Reverse primer | Amplicon size |
| *acsl4* | GTCATTTTGGGAGCGGACTG | GTGACCAAGGGGAAGTTGC | 130 |
| *cep135* | CAGACTCAAGGTTGCTCACAGC | AACCACACGACTTTCCTTCAGC | 151 |
| *dnmt3ba* | ATGGACTTCTGCCCTTTGG | TACCTCTTCGCCTTTCTTCTC | 158 |
| *golga7bb* | GCGAGTGGAGAGGATTTCAG | AGCTCCTGAAGGTTGTGGAA | 85 |
| *hlfb* | GCACCAAGATGAAGGCTATGG | AAGCAGACTGCGGAGGGTTT | 80 |
| *insig1* | GGTCTACAACGGCATCTACCA | TTCTCAGTCGAGCCCATAGC | 135 |
| *lin7a* | GGAGCTGCCCAAGACAGATG | TCACCCTCAACGCTCACTCC | 171 |
| *rpgrb* | CCCACTTGTGTAAAAGCTCTGAAG | TCCCTCCACAGGCATACAGG | 109 |
| *rpl31* | GATGAGGATTCCCCAAACAA | TTACGTCCGCATGTCAACAC | 188 |
| *rps12* | CGCAGTCAGAGGGAAGGTAG | AGAGCGGTGTTGACATCCAT | 230 |
| *sp9* | GGCTACGTCTATACTTGGGGAAGAG | TGCCAATTTTGTTGCAGGTC | 83 |
| *ifi45* | CAAAACACTTCGGTGGCTTT | TGGATGATCTTGCTCTGCAC | 115 |
| *kdm6bb* | GATCCCTCCGTCTCAATCTGG | CTGGGTCCTCACCTCCACTG | 86 |
| *scn1lab* | ATCTGTGCCAACATCACCAA | CTCCTTCACCTTCAGCCAAG | 271 |
| *rnft2* | TGCCACCAGAAAGGAACAGG | CTGGGGAGACGTGGGAGAG | 104 |
| *arnt* | GTCGGTGATGTTTCGCTTCC | CTGCAACTGCTTGACGTTGG | 136 |
| *lrrmtl41l* | TATGGGTTCTCTTGCGTGTG | CACATTCGTTCCCCAGAACT | 93 |
| *fosaa* | AGAACCGACAGCAATGAACC | CTCTCGATTTCAGCCTGGAG | 136 |
| *b2m* | ACAGGGGAAAGTCTCCACTCCGAA | AGGTCGGTCTGCTTGGTGTCC | 168 |
| *rpl13a* | TCTGGAGGACTGTAAGAGGTATGC | AGACGCACAATCTTGAGAGCAG | 164 |

## **Table S4**: Comparison of fold-changes obtained from RNA-Seq and qPCR performed on different samples (biological and technical validation). \*: removed from correlation analysis.

|  |  |  |
| --- | --- | --- |
|  | qPCR | RNA-Seq |
| ***F0\_M*** |
| *acsl4* | 0.82 | 0.69 |
| *cep135* | 0.79 | 0.25 |
| *dnmt3ba* | 0.86 | 0.66 |
| *golga7bb* | 0.76 | 0.27 |
| *hlfb* | 0.90 | 0.47 |
| *insig1* | 0.82 | 0.67 |
| *lin7a* | 0.61 | 0.65 |
| *rpgrb* | 0.54 | 0.48 |
| *rpl31* | 0.78 | 1.39 |
| *rps12* | 0.85 | 1.53 |
| *sp9* | 0.65 | 1.39 |
| *ifi45* | 1.55 | 3.23 |
| *kdm6bb* | 0.84 | 0.43 |
| *scn1lab* | 0.70 | 0.59 |
| ***F2\_M*** |
| *acsl4a* | 0.83 | 0.41 |
| *cep135* | 0.80 | 0.17 |
| *hlfb* | 1.10 | 168.90\* |
| *insig1* | 1.19 | 0.45 |
| *lin7a* | 1.22 | 0.77 |
| *rpgrb* | 1.24 | 0.91 |
| *rpl31* | 0.96 | 0.71 |
| *rps12* | 1.27 | 0.68 |
| *rnft2* | 1.04 | 0.54 |
| *arnt* | 1.09 | 1.61 |
| *lrrmtl41l* | 1.08 | 2.14 |
| ***F2\_F*** |
| *lin7a* | 1.02 | 0.52 |
| *rpgrb* | 1.82 | 0.21 |
| *rnft2* | 1.48 | 0.58 |
| *arnt* | 1.62 | 5.81 |
| *lrrtm4l1* | 1.58 | 2.25 |
| *fosaa* | 1.67 | 7.73 |

# **Section S4**: RNA-Sequencing

## **Table S5**: List of commonly significantly regulated genes in F0 and F2 males. Complete lists of differentially expressed genes per generation are available in the Excel file.

|  |  |  |  |
| --- | --- | --- | --- |
| Gene | Description | Log2FC F0 Male | Log2FC F2 male |
| *cep135* | centrosomal protein 135 | -2.03 | -2.56 |
| *acsl4a* | acyl-CoA synthetase long chain family member 4a | -0.53 | -1.30 |
| *insig1* | insulin induced gene 1 | -0.59 | -1.16 |
| *timd4* | T cell immunoglobulin and mucin domain containing 4 | 0.50 | -1.01 |
| *calr* | calreticulin | -0.62 | -0.89 |
| *ndufb3* | NADH:ubiquinone oxidoreductase subunit B3 | 0.48 | -0.77 |
| *naca* | nascent polypeptide associated complex subunit alpha | -0.35 | -0.67 |
| *rps28* | ribosomal protein S28 | 0.43 | -0.58 |
| *rps12* | ribosomal protein S12 | 0.61 | -0.56 |
| *rpl31* | ribosomal protein L31 | 0.48 | -0.51 |
| *tapbp.2* | TAP binding protein (tapasin), tandem duplicate 2 | 1.45 | 2.08 |
| *hlfb* | HLF transcription factor, PAR bZIP family member b | -1.10 | 7.40 |

## **Table S6**: List of energy-related GO and REACTOME gene sets significantly regulated in F0 brains of male zebrafish after exposure to MIX. Complete lists of enriched GO and REACTOME gene sets per generation are available in the Excel file.

|  |  |  |  |
| --- | --- | --- | --- |
| **ID** | **Description** | **Enrich. Score** | **p-adj** |
| REAC:R-DRE-9020702 | Interleukin-1 signaling | 1.71 | 0.033 |
| REAC:R-DRE-70263 | Gluconeogenesis | 1.71 | 0.0329 |
| GO:0006090 | pyruvate metabolic process | 1.86 | 0.0099 |
| REAC:R-DRE-71406 | Pyruvate metabolism and Citric Acid (TCA) cycle | 2.04 | 0.0014 |
| REAC:R-DRE-163210 | Formation of ATP by chemiosmotic coupling | 2.44 | 0 |
| REAC:R-DRE-163200 | Respiratory electron transport, ATP synthesis by chemiosmotic coupling, and heat production by uncoupling proteins. | 3.08 | 0 |
| REAC:R-DRE-1428517 | The citric acid (TCA) cycle and respiratory electron transport | 3.06 | 0 |
| GO:0003954 | NADH dehydrogenase activity | 2.34 | 0 |
| GO:0030964 | NADH dehydrogenase complex | 2.78 | 0 |
| GO:0017004 | cytochrome complex assembly | 2.39 | 0 |
| REAC:R-DRE-611105 | Respiratory electron transport | 2.85 | 0 |
| GO:0009055 | electron carrier activity | 2.58 | 0 |
| GO:0098803 | respiratory chain complex | 3.01 | 0 |
| GO:0070469 | respiratory chain | 3.03 | 0 |
| GO:0098798 | mitochondrial protein complex | 3.06 | 0 |
| GO:0006839 | mitochondrial transport | 1.97 | 0.0033 |
| GO:0045333 | cellular respiration | 2.42 | 0 |

## **Table S7**: List of neurotransmission-related GO and REACTOME gene sets significantly regulated in F0 and F2 brains of male zebrafish after exposure to MIX. Complete lists of enriched GO and REACTOME gene sets per generation are available in the Excel file.

|  |  |  |  |
| --- | --- | --- | --- |
| **ID** | **Description** | **Enrich. score F2** | **p-adj** |
| REAC:R-DRE-6794361 | Neurexins and neuroligins | 1.76 | 0.0493 |
| REAC:R-DRE-212676 | Dopamine Neurotransmitter Release Cycle | 1.98 | 0.0139 |
| REAC:R-DRE-1296072 | Voltage gated Potassium channels | 1.90 | 0.0256 |
| GO:0050808 | synapse organization | 1.77 | 0.0471 |
| GO:0035418 | protein localization to synapse | 1.87 | 0.0297 |
| GO:0097120 | receptor localization to synapse | 1.91 | 0.0233 |
| GO:0036269 | swimming behavior | 1.98 | 0.0134 |
| GO:0099072 | regulation of postsynaptic specialization membrane neurotransmitter receptor levels | 2.00 | 0.012 |
| GO:0034703 | cation channel complex | 2.03 | 0.0094 |
| GO:0044325 | ion channel binding | 1.85 | 0.0298 |
| GO:0051966 | regulation of synaptic transmission, glutamatergic | 2.06 | 0.0064 |
| GO:0048489 | synaptic vesicle transport | 2.14 | 0.0035 |
| GO:0099637 | neurotransmitter receptor transport | 2.16 | 0.0025 |
| GO:0008066 | glutamate receptor activity | 2.21 | 0.0017 |
| GO:0022843 | voltage-gated cation channel activity | 2.22 | 0.0015 |
| GO:0014069 | postsynaptic density | 2.26 | 0.001 |
| GO:0007216 | G-protein coupled glutamate receptor signaling pathway | 2.27 | 0.0015 |
| **ID** | **Description** | **Enrich. score F0** | **p-adj** |
| REAC:R-DRE-442755 | Activation of NMDA receptors and postsynaptic events | -1.82 | 0.0479 |
| REAC:R-DRE-112315 | Transmission across Chemical Synapses | -1.89 | 0.0381 |
| GO:0032590 | dendrite membrane | -2.21 | 0.0023 |
| GO:0016917 | GABA receptor activity | -2.20 | 0.0017 |
| GO:0007214 | gamma-aminobutyric acid signaling pathway | -2.08 | 0.0086 |
| GO:0003774 | motor activity | -1.99 | 0.0228 |
| GO:0001518 | voltage-gated sodium channel complex | -1.99 | 0.0228 |
| GO:0048017 | inositol lipid-mediated signaling | -1.97 | 0.0196 |
| GO:0048015 | phosphatidylinositol-mediated signaling | -1.94 | 0.0261 |
| GO:0008308 | voltage-gated anion channel activity | 1.72 | 0.0323 |

## **Table S8**: List of GO and REACTOME gene sets significantly regulated in both F0 and F2 brains of male zebrafish after exposure of F0 to MIX. Complete lists of enriched GO and REACTOME gene sets per generation are available in the Excel file.

|  |  |  |  |
| --- | --- | --- | --- |
| ID | Description | Enrich. Score F0 | Enrich. Score F2 |
| ***RNA regulation/Transcription and translation machinery (17)*** |
| GO:0003735 | structural constituent of ribosome | 3.27 | -3.54 |
| GO:0005840 | ribosome | 3.25 | -3.54 |
| REAC:R-DRE-975956 | Nonsense Mediated Decay (NMD) independent of the Exon Junction Complex (EJC) | 3.25 | -3.31 |
| REAC:R-DRE-72706 | GTP hydrolysis and joining of the 60S ribosomal subunit | 3.21 | -3.35 |
| REAC:R-DRE-975957 | Nonsense Mediated Decay (NMD) enhanced by the Exon Junction Complex (EJC) | 3.17 | -3.26 |
| REAC:R-DRE-927802 | Nonsense-Mediated Decay (NMD) | 3.15 | -3.24 |
| REAC:R-DRE-1799339 | SRP-dependent cotranslational protein targeting to membrane | 3.14 | -3.42 |
| REAC:R-DRE-72613 | Eukaryotic Translation Initiation | 3.08 | -3.28 |
| REAC:R-DRE-72737 | Cap-dependent Translation Initiation | 3.03 | -3.34 |
| REAC:R-DRE-72766 | Translation | 2.92 | -3.12 |
| REAC:R-DRE-72702 | Ribosomal scanning and start codon recognition | 2.44 | -2.97 |
| REAC:R-DRE-72695 | Formation of the ternary complex, and subsequently, the 43S complex | 2.44 | -2.98 |
| GO:0002181 | cytoplasmic translation | 2.23 | -2.88 |
| REAC:R-DRE-72165 | mRNA Splicing - Minor Pathway | 2.18 | -1.84 |
| GO:0019843 | rRNA binding | 2.06 | -2.39 |
| GO:0000786 | nucleosome | 1.89 | 1.89 |
| GO:0044815 | DNA packaging complex | 1.80 | 1.86 |
| ***Mitochondria (6)*** |
| GO:0016675 | oxidoreductase activity, acting on a heme group of donors | 2.51 | -1.96 |
| REAC:R-DRE-5389840 | Mitochondrial translation elongation | 2.50 | -2.18 |
| GO:0044445 | cytosolic part | 3.13 | -2.94 |
| GO:0015002 | heme-copper terminal oxidase activity | 2.49 | -1.93 |
| REAC:R-DRE-5368287 | Mitochondrial translation | 2.42 | -2.22 |
| REAC:R-DRE-5419276 | Mitochondrial translation termination | 2.40 | -2.16 |
| ***Proteasomal activity (3)*** |
| GO:0000502 | proteasome complex | 2.47 | -1.83 |
| GO:0004298 | threonine-type endopeptidase activity | 2.16 | -1.88 |
| GO:0070003 | threonine-type peptidase activity | 2.09 | -1.86 |
| ***Neurotransmission (1)*** |
| REAC:R-DRE-6794362 | Protein-protein interactions at synapses | -1.83 | 1.85 |
| ***Unclassified*** |
| REAC:R-DRE-5625740 | RHO GTPases activate PKNs | 2.39 | 1.78 |
| GO:0017001 | antibiotic catabolic process | 1.81 | 1.96 |
| GO:0007156 | homophilic cell adhesion via plasma membrane adhesion molecules | -1.84 | 1.87 |

## **Table S9**: List of GO and REACTOME gene sets significantly regulated in both F2 females and F0 or F2 males. A total of 27 significantly enriched terms was reported in F2 females, mainly related to mitochondrial respiration, cell cycle, and protein metabolism. Up to 63% (17/27) of these gene sets were already significantly regulated in F0 MIX males. Complete lists of enriched GO and REACTOME gene sets per generation are available in the Excel file.

|  |  |  |  |
| --- | --- | --- | --- |
| ID | Description | NES (F2\_M/F0\_M) | NESF2\_F |
| ***Gene sets regulated in F2\_M and F2\_F*** |
| GO:0051966 | regulation of synaptic transmission, glutamatergic | 2.06 | 1.92 |
| GO:0000502 | proteasome complex | -1.83 | 2.22 |
| S4REAC:R-DRE-450282 | MAPK targets/ Nuclear events mediated by MAP kinases | 1.78 | 2.01 |
| GO:0004722 | protein serine/threonine phosphatase activity | 2.11 | 2.10 |
| ***Gene sets regulated in F0\_M and F2\_F*** |
| GO:0000502 | proteasome complex | 2.47 | 2.22 |
| GO:0008287 | protein serine/threonine phosphatase complex | 1.66 | 2.10 |
| GO:0030964 | NADH dehydrogenase complex | 2.78 | 1.98 |
| GO:0040036 | regulation of fibroblast growth factor receptor signaling pathway | 1.99 | 1.96 |
| GO:1903293 | phosphatase complex | 1.65 | 2.14 |
| GO:0006900 | membrane budding | 1.69 | 2.05 |
| REAC:R-DRE-163200 | Respiratory electron transport, ATP synthesis by chemiosmotic coupling, and heat production by uncoupling proteins. | 3.08 | 1.92 |
| REAC:R-DRE-163210 | Formation of ATP by chemiosmotic coupling | 2.44 | 1.92 |
| REAC:R-DRE-174084 | Autodegradation of Cdh1 by Cdh1:APC/C | 2.59 | 2.27 |
| REAC:R-DRE-174143 | APC/C-mediated degradation of cell cycle proteins | 2.62 | 2.16 |
| REAC:R-DRE-2173793 | Transcriptional activity of SMAD2/SMAD3:SMAD4 heterotrimer | 2.01 | 1.90 |
| REAC:R-DRE-3301854 | Nuclear Pore Complex (NPC) Disassembly | 2.02 | 1.92 |
| REAC:R-DRE-453276 | Regulation of mitotic cell cycle | 2.67 | 2.19 |
| REAC:R-DRE-68827 | CDT1 association with the CDC6:ORC:origin complex | 2.48 | 2.35 |
| REAC:R-DRE-68867 | Assembly of the pre-replicative complex | 2.56 | 2.23 |
| REAC:R-DRE-69002 | DNA Replication Pre-Initiation | 2.46 | 2.07 |
| REAC:R-DRE-69306 | DNA Replication | 2.36 | 1.96 |

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