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# A multiscale approach to decipher the molecular mechanisms involved in the direct and intergenerational effect of ibuprofen on the mosquito *Aedes aegypti.*

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**ABSTRACT:**

The anti-inflammatory ibuprofen is a ubiquitous surface water contaminant. However, the chronic impact of this pharmaceutical on aquatic invertebrate population remains poorly understood. In the model insect *Aedes aegypti*, we investigated the intergenerational consequences of parental chronic exposure to an environmentally relevant concentration of ibuprofen. While exposed individuals did not show any phenotypic changes, their progeny showed accelerated development and an increased tolerance to starvation. In order to understand the mechanistic processes underpinning the direct and intergenerational impacts of ibuprofen, we combined transcriptomic, metabolomics and hormone kinetics studies at several life-stages in exposed individuals and their progeny. This integrative approach revealed moderate transcriptional changes in exposed larvae consistent with the pharmacological mode of action of ibuprofen. Parental exposure led to lower levels of several polar metabolites in progeny eggs, and to major transcriptional changes at the following larval stage. These transcriptional changes, most likely driven by changes in expression of numerous transcription factors and epigenetic regulators, led to ecdysone signalling and stress response potentiation. Overall, the present study illustrates the complexity of the molecular basis of the intergenerational pollutant response in insects and the importance of considering the entire life cycle of exposed organisms and of their progeny, in order to fully understand the mode of action of pollutants and their impact of ecosystems.

34 **INTRODUCTION:**

35 Since the early 2000s, the worldwide surface water contamination by pharmaceutical products and the associated
36 ecotoxicological consequences on aquatic organisms has been an issue of increasing concern.[1,2] Ibuprofen, a
37 non-steroidal anti-inflammatory drug used for general pain relief, is one of the most commonly detected
38 pharmaceutical products.[3,4] Due to its widespread use, ibuprofen is chronically released into the aquatic
39 environment, via urinary excretion and the direct disposal of unused or outdated pills in urban wastewater.[2]
40 Despite its relatively short life-time in water,[5] the continuous release of ibuprofen causes a phenomenon of
41 pseudo-persistence in aquatic ecosystems.[6] This results in measured concentrations usually ranging from 0.005
42 to 5 µg/L in European surface water, up to tens of µg/L at several sites worldwide.[3,4,7–10] Several studies have
43 illustrated the impact of chronic exposure to ibuprofen at individual or sub-individual level on several freshwater
44 invertebrates such as mollusks, cnidarians and crustaceans.[11–17] Hence, the occurrence, persistence and
45 ecotoxicological data currently available for this pharmaceutical product have led several authors to classify
46 ibuprofen as a priority pollutant for ecotoxicological risk assessment.[18,19]

47 In mammals, the anti-inflammatory effect of ibuprofen is due to an inhibition of prostaglandin synthesis through
48 competitive fixation on a cyclooxygenase (COX) active site.[20] Prostaglandins belong to the eicosanoids family
49 and are messengers of the inflammatory reaction. These lipid compounds are synthetized from arachidonic acid
50 oxidation by COX1 and COX2.[20] This mode of action (MOA) has also been demonstrated in freshwater fish
51 exposed to ibuprofen.[21,22] Surprisingly, the molecular and physiological alterations induced in ibuprofen-exposed
52 invertebrates remain poorly documented, despite the fact that invertebrates are usually remarkably abundant in
53 water ecosystems and are an important part of the food web. Acute exposure of *Daphnia magna* led to the
54 modification of expression of several enzymes involved in eicosanoids synthesis, and was associated with
55 impaired reproductive function.[23] Similarly, Milan *et al.* demonstrated that exposure of the manila clam
56 *Ruditapes philippinarum* to concentrations ranging from 0.1 to 1 mg/L over 7 days modifies the transcription
57 rate of several genes involved in cell respiration, arachidonic acid metabolism, eicosanoid metabolism, immunity
58 and inflammatory response.[24] It is important to note that these transcriptomic-based studies were performed at
59 unrealistic exposure concentrations. As the transcriptomic responses elicited by a pollutant largely depend on the
60 exposure concentration, mechanistic studies performed at high concentrations may not be representative of the
61 transcriptional modifications elicited in organisms at environmental concentrations.[21] A recent study on the
62 freshwater mussel *Dreissena polymorpha* using more environmentally realistic concentrations demonstrated that

acute exposure induced decreased COX activity at concentrations ranging from 10 to 100 µg/L, though the molecular mechanisms involved in the resulting physiological changes were not assessed in this study.[25]

Understanding the inter-, trans- and multigenerational impact of pollutants is an area of increasing interest and constitutes a major challenge in ecotoxicology, as it allows a better grasp of the long-term consequences of the chronic exposure of populations to pollutants.[26–30] In a recent study, we evaluated the short and long-term impact of chronic exposure to an environmentally-relevant concentration of ibuprofen (1µg/L) on the life-history traits of the *A. aegypti* mosquito.[28] Although no changes were observed in exposed F0 individuals, this work demonstrated transgenerational consequences of ibuprofen exposure, leading to an increased developmental rate in the F1 progeny grown in an unpolluted environment. As a vector of several arboviruses responsible for major human diseases such as dengue fever, yellow fever, chikungunya or zika, a huge amount of ecological, physiological and genomic data are available for this mosquito species. In particular, the hormonal and metabolic regulation of key developmental and reproductive events have been extensively characterized.[31–33] Its genome has been sequenced and continuously annotated since 2007, leading to an improved understanding of the molecular basis of its physiology over the past 10 years.[34] This insect species thus represents a valuable biological model for delineating the interactions between genotypic and phenotypic responses in aquatic insects, and thus improving our understanding of the impacts of pollutants on individuals.

In this context, the present study aims at understanding the molecular basis of the direct and intergenerational disturbances caused by ibuprofen exposure in *A. aegypti,* and how these link to phenotype modifications. We developed an integrative approach combining lifespan and stress tolerance measurement with metabolomics, transcriptomics, and hormone kinetics to assess the general physiological state of directly-exposed individuals and their progeny. As well as providing new insights into the direct consequences of ibuprofen exposure, this approach allowed us to understand the molecular basis of the disturbed phenotype in the progeny of exposed individuals.

## METHODS:

### Animal rearing

A laboratory strain of *A. aegypti* (Liverpool strain) was provided by the Pasteur institute (Paris, France) and used for all experiments. The rearing conditions used for aquatic stages and imagoes (*i.e.* mature life stage in insects) were the same as in Prud'homme *et al,*[28] and are detailed in SI-1.

### Chronic exposure of mosquitoes to ibuprofen

Ibuprofen (purity 98%, CAS no. 15687-27-1), was purchased from Sigma–Aldrich (St. Louis, MO). Ten mg/L stock solutions were prepared in absolute ethanol (HPLC grade) at the beginning of the experiment and aliquots were stored at -20°C. The contaminated media was freshly prepared with tap water and aliquots of ibuprofen stock solution to reach concentrations of 1 µg/L. The final solvent (ethanol) concentration was 0.002% for the contaminated and control media. Chronic exposure to ibuprofen or solvent-control, lasting from larvae hatching to imagoes emergence, was conducted with static renewal of the media, replaced every 48h. F1 spawn obtained from F0 exposed and control individuals were raised in unpolluted media.

In order to ensure effective concentrations of ibuprofen in the culture media, the contaminated media were freshly prepared in two separate glass dishes and kept in the same light and temperature conditions as in our experiment. Larvae were added to one of the dishes at a density of 1 larva/mL to compare the change in ibuprofen concentration in the culture media, with or without the presence of larvae. 500 mL aliquots were sampled at 0, 24, and 48h (without larvae) and at 48h (with larvae) and stored at -20 °C until ibuprofen concentration measurement. Ibuprofen was extracted by solid phase extraction and measured by LC-HRMS/MS (SI-2). Dosage accuracy was determined by measuring concentration of analytical standard in five independent extraction replicates of a reference media with 1µg/L of analytical standard. Initial concentrations of ibuprofen were close to the nominal concentration (1 µg/L) and remained relatively stable over 48h, with or without larvae (Figure S1).

**Experimental design**

All F0 and F1 populations were raised at a density of 1 larvae/mL, in the same light, temperature and food conditions as described in SI-1. In these conditions, imago emergence occurred from the 7$^{th}$ (males) and 9$^{th}$ (females) days after hatching. In *Aedes aegypti*, mating occurs immediately after female emergence, and females are mature to spawn 72h after emergence. Egg maturation is initiated by blood feeding, and eggs are spawned on the 3rd day following the blood-meal. In our experiment, a blood-meal was provided to F0 females 15 days after the start of our experiment (1$^{st}$ stage larvae hatching), to ensure their mating status and sexual maturity. Egg fertilization and embryonic development are concomitant to spawning, and dried eggs enter into a quiescent phase at the end of embryonic development. The hatching of quiescent eggs occurs in the 1$^{st}$ hour following egg immersion.

In a first experiment, populations of 2,000 individuals were either exposed to ibuprofen and solvent or solvent only (controls). Experiments were performed in quadruplicate concomitantly for both ibuprofen exposure and

controls, and all replicates were raised in parallel. Individuals were collected at different time points, corresponding to different developmental stages in exposed populations (F0) and their progeny subsequently grown in unpolluted medium (F1) (Figure S2). These individuals were further used to perform individual **phenotypic characterization** (Figure S2: ③, ⑪), to monitor **hormone levels** and the **transcription level of key associated genes during egg maturation** (Figure S2: ⑦, ⑧) and to perform a **transcriptome-wide gene expression study by RNA-seq** (Figure S2: ①, ⑥, ⑩). In a second experiment, two independent sets of 2,000 individuals were exposed to ibuprofen or solvent alone (control) in the same conditions detailed above, and individuals were collected at several developmental stages to perform a comparative **directed metabolomic analysis by GC-MS** (Figure S2: ②, ④, ⑤, ⑨).

## Phenotypic responses to chronic exposure

### *2.4.1 Imago lifespan*

F0 imagoes from each replicate population were collected in a 10-hour window after emergence. Females and males were separated before mating and placed in distinct cages in order to assess the lifespan of virgin individuals (3 replicate cages per condition). A total of at least 59 virgin males and 32 virgin females were isolated from ibuprofen exposed and control populations respectively. At least 52 primiparous females from each F0 population replicate were collected one day after their first egg-laying and placed in separate cages. Primiparous females emerged in a time laps of 4 days. The lifespan of these females is thus considered ± 2 days. Imagoes had access to water and sucrose *ad libitum*. The number of dead individuals in each cage was assessed daily until the death of the last surviving imago.

### *2.4.2 Progeny tolerance to starvation*

The emerging imagoes remaining after sampling for lifespan measurement, hormonal and transcriptional analysis were collected twice a day and males and females were placed in net cages for mating, as previously described.[28] F1 eggs spawned after the first blood-meal were collected and air-dried for one week. F1 egg batches (≈ 1000 eggs) were then simultaneously immersed to elicit hatching as described.[28] For each replicate population, 3 groups of 20 newly hatched larvae (< 1h) were isolated, resulting in 12 groups per parental exposure condition. These larvae were raised in unpolluted media with no food resources in order to assess starvation-survival in the F1 progeny. Surviving larvae were counted daily until the death of the last surviving larvae. Dead individuals were regularly removed to avoid necrophagy. Larvae that did not respond to contact stimulation were considered dead.

150 *2.4.3 Statistical analysis*

151 The survival curves for F0 imagoes exposed to ibuprofen or solvent-control and of their F1 larvae progeny were
152 compared using a Kaplan-Meyer survival test performed with R software.[35]

153 **Ecdysteroid dynamics during vitellogenesis**

154 Four females were collected from each F0 replicate population 30 min before and 4, 8, 16, 20, 24, 36 and 48h
155 after blood feeding in order to assess ecdysteroid titer dynamics during vitellogenesis (Figure S2, ⑧). The
156 collected females were isolated in 250 µL methanol (MeOH) pro-analysis (Merk, Germany) and stored at -20°C.
157 Total ecdysteroids from whole animals were extracted with MeOH and re-dissolved in enzymatic immunoassay
158 (EIA) buffer (SI-3). Ecdysteroids were quantified using an EIA adapted from the method described in Porcheron
159 *et al.*[36] (SI-3). Ecdysteroids titers were estimated using a 20-hydroxyecdysone (20E) calibration curve (8 to 4,000
160 fmol; Sigma-Aldrich, USA) diluted in EIA reaction buffer, and titers were expressed as 20E equivalents. All
161 measurements were performed in duplicate and the results were expressed as mean values ± SEM. Eight to 16
162 females were assessed at each time-point for each exposure condition. Mean ecdysteroid titer at each time-point
163 was compared between exposed and control females using a Student's t-test, after data checking for normality
164 (Shapiro-Wilk test) and homoscedasticity (F-test). The resulting p-values were corrected for multiple comparison
165 (8 time-points) using the FDR method.

166 **Gene transcription dynamics during vitellogenesis**

167 The transcription levels of 5 ecdysone related genes (*74EF*, *VtgA*, *VtgR*, *Lp*, *LpRov*, Table S1) were assessed at
168 several stages in the vitellogenic process. Four pools of 5 to 10 blood-fed females were collected from each F0
169 replicate 30 min before and 4h, 8h, 16h, 20h, 24h, 36h and 48h after blood feeding and stored in TRIzol
170 (Invitrogen Life Technologies, USA) at -80°C. Total RNAs were extracted from each sample (SI-4) and real-
171 time quantitative PCR (RT-qPCR) reactions were performed in triplicate on the iQ5 system (Bio-Rad, USA) (SI-
172 4). The primer sequences are listed in Table S1. The primers used for RT-qPCR were designed using Beacon
173 Designer™ 5.10 software, except for the rpl32, VtgR and LpRov genes, for which the primers were already
174 published.[37–39] Data analysis was performed according to the ΔΔCt method,[40] taking into account PCR efficiency
175 and using the housekeeping genes encoding the ribosomal protein RPL32 (AAEL00339) and actin
176 (AAEL001673) for dual-gene normalization. The results were expressed as the mean transcription ratio between
177 ibuprofen exposed and control females at each time point. Wilcoxon tests were performed to estimate the

178 significant changes at each gene transcription level between ibuprofen-exposed and control females at each time
179 point. The resulting p-values were corrected for multiple comparison (8 time-points) using the FDR method.

180 **Metabolomic GC-MS analysis**

181 The metabolomic experiments were performed on F0 4$^{th}$ stage larvae (L4F0), F0 male and female imagoes
182 (respectively MalF0 and FemF0), and F1 eggs 4h after laying (EggF1), obtained from the two population
183 replicates exposed to ibuprofen or the two control populations. Three batches of 10 4$^{th}$ stage F0 larvae (34-40
184 hours after moult) were collected from each replicate of both exposure conditions. Four batches of 10 virgin F0
185 females and 4 batches of 10 virgin F0 males were collected 72h after emergence from each replicate of both
186 exposure conditions. Batches of individuals were also sampled for cytosolic protein content analysis (SI-5).
187 Following the F0 population breeding, three batches of 100 F1 eggs were collected in the 4 hours following egg-
188 laying from each replicate of both exposure conditions. Samples were snap frozen in liquid $N_2$ before being
189 stored at -80°C. The samples were then lyophilized, weighed, and stored at -20°C until metabolite extraction
190 using methanol/chloroform/water (2/1/2) solid/liquid extraction. The detailed extraction and derivatization
191 procedure is provided in the supplementary information (SI-6.1/2). The metabolomics approach was directed
192 toward 53 polar metabolites representatives of the insect metabolome (Table S2). Metabolite quantification was
193 performed using Gas Chromatography-Mass Spectrometry (GC-MS) on a system consisting of a CTC CombiPal
194 autosampler (PAL System, CTC Analytics AG, Zwingen, Switzerland), a Trace GC Ultra chromatograph and a
195 Trace DSQII quadrupole mass spectrometer (Thermo Fischer Scientific Inc, Waltham, MA, USA). The detailed
196 quantification procedure is provided in the supplementary information (SI-6.3). Metabolite concentrations were
197 expressed as nmol/mg of dry mass. Concentration data were imported in Metaboanalyst 3.0 for the statistical
198 analyses.[41] Data were paretoscaled before the statistical analyses and individual metabolite concentrations were
199 compared according to the exposure group using a multivariate approach by PCA and supervised orthogonal
200 partial least square discriminant analyses (OPLS-DA) (SI-6.4). Individual concentrations of the metabolites were
201 compared using Wilcoxon rank-sum test. The resulting p-values were adjusted using Benjamini & Hochberg's
202 method (FDR).

203 **Transcriptomic analysis by RNAseq**

204 The transcriptomic analysis was performed on F0 4$^{th}$ stage larvae (L4F0), F0 imago females (FemF0) and 1$^{st}$
205 stage larvae from F1 progeny (L1F1). In each of the four F0 biological replicate populations, batches of 35
206 exposed or control 4$^{th}$ stage larvae were collected 34h to 40h following moult to perform the transcriptomic

207 analysis (Figure S2, ①). Batches of 30 mature virgin females, raised with *ad libitum* access to water and 10%
208 sucrose, were collected 72 hours after emergence in each F0 independent replicate, after being instantly killed by
209 brief immersion in 70% ethanol (Figure S2, ⑥). Batches of 300 newly-hatched F1 larvae (< 30 min) spawned
210 by each F0 replicate were reared in unpolluted medium with food. These larvae were collected 10h after
211 hatching for the transcriptome analysis of the unexposed progeny (F1) (Figure S2, ⑩). Collected L4F0 samples
212 were immediately stored in TRIzol reagent (Invitrogen Life Technologies, USA) at -80°C. FemF0 and L1F1
213 samples were immediately immerged in RNAlater (Ambion, USA) and stored at 4°C for 24h, and then at -80°C
214 before RNA extraction. The procedure for total RNA extraction is detailed in the supporting information (SI-
215 7.1). Out of the four independent RNA samples collected for each life-stage and each exposure condition, the
216 three showing the best quality profile on BioAnalyseur 2100 (Agilent Technologies, USA) were used for the
217 high throughput RNA sequencing (RNAseq) transcriptomic analysis. c-DNA libraries were prepared from 400ng
218 total RNA using Illumina TruSeq Stranded mRNA Sample Preparation kits (Illumina Inc., USA) following the
219 manufacturer's instructions. Libraries were sequenced as 75 bp single reads (L4F0) or 150 bp single reads
220 (FemF0 and L1F1) on an Illumina platform by Helixio-Hybrigenics, France.

221 Raw-data processing was performed on the galaxy.prabi.fr web service and the computing facilities of the
222 LBBE/PRABI (Lyon, France). Data were first filtered according to size and quality parameters using
223 Trimmomatic[42] (SI-7.2). Tophat2[43] (v0.6) was used to map filtered reads to the *A. aegypti* reference genome
224 (Aaeg L3.3 assembly and gene set, http://vectorbase.org). The following data processing was based on the
225 Cufflink RNA-seq workflow (v2.2.0; http://cole-trapnell-lab.github.io/ cufflinks/),[44,45] using the Cufflink,
226 Cuffmerge and Cuffdiff suite of tools to identify differentially transcribed genes (DTG) between ibuprofen and
227 control individuals (L4F0, FemF0) or progeny (L1F1) (SI-7.2). Genes were considered as differentially
228 transcribed when the transcription ratio TR (ibuprofen vs. control) was >1.2 in either direction, with a corrected
229 p-value (q-value; Benjamini-Hochberg correction) below 0.05. Only genes for which transcription level was
230 superior to 0.5 FPKM in at least one of the two considered conditions were considered, in order to avoid
231 expression level inaccuracy. The median transcriptional fold-changes for each transcriptomic analysis were
232 compared using a Kruskal-Wallis test followed by a Dunn's test, using FDR to adjust the p-values.
233 For the functional analysis, 20 functional classes were defined to help describe the physiological processes
234 affected by ibuprofen exposure, and all detected genes were assigned to one these classes based on their
235 annotation (see Table S5 and SI). DTG repartition among these functional classes was compared to the
236 distribution of all detected genes using a Fisher's exact test where p-values were corrected for multiple-testing

237 using the FDR method. The enrichment of some functional classes among DTG was studied independently for
238 up-regulated genes and down-regulated genes.

239 **RESULTS AND DISCUSSION**

240 **Phenotypic consequences of direct and parental ibuprofen exposure**

241 Direct ibuprofen exposure did not impact the lifespan of virgin and primiparous F0 females while F0 virgin male
242 imago showed a 23% increased lifespan (Xsq=3.8, df=1, p-value=0.051) (Figure 1). Our previous study revealed
243 that ibuprofen exposure did not affect the development of F0 individuals or imago size, suggesting that ibuprofen
244 exposure has limited phenotypic consequences on F0 individuals.[28] In F1 progeny, the survival of 1$^{st}$ stage larvae
245 obtained from exposed parents under starving condition was doubled compared to the controls (Xsq=18.4, df=1,
246 p-value=1.8e$^{-05}$) (Figure 2). These results, combined with the reduction in development time previously observed
247 in the F1 progeny of ibuprofen-exposed individuals (Figure S3), confirms the phenotypic changes caused by
248 chronic parental exposure to ibuprofen.

249 **Overall transcriptomic consequences of direct and parental ibuprofen exposure**

250 The transcriptomic analyses identified 134 differentially transcribed genes (DTGs) in F0 4$^{th}$ stage larvae (L4F0),
251 40 DTGs in F0 female imagoes (FemF0) and 1566 DTGs in F1 1$^{st}$ stage larvae (L1F1) between exposed
252 individuals and the controls respectively. This represents 1.1%, 0.3% and 14.8% of the genes detected at each of
253 the three life-stages studied (Figure 3, A). DTG were poorly shared among the developmental stages (Figure 3,
254 B). Interestingly, the range of differential transcription was greater in directly exposed individuals as compared
255 to their progeny, with median fold-changes associated to DTG that were greater in L4F0 (z=-6.27, p-value≈0)
256 and FemF0 (z=9.03, p-value≈0) than in L1F1 (Figure 3, C). As most phenotypic variations were observed in the
257 F1 progeny of exposed individuals (increased starvation-survival and faster development), the range of
258 differential transcription may not necessarily be associated with evident phenotypic changes at the individual
259 scale. Conversely, numerous but moderate transcriptional changes can cause a pronounced disruption in
260 development, as previously demonstrated in *Daphnia magna*.[46]

261 **Molecular changes associated with direct ibuprofen exposure in 4$^{th}$ stage F0 larvae**

262 Direct exposure did not affect the representation of the 53 quantified polar metabolites in 4$^{th}$ stage F0 larvae
263 (Figure S4, A, Table S6), but induced the differential transcription of 134 genes. Interestingly, the transcriptomic

264 profile analysis did not show any stress-response related markers. Among the DTGs, 51 genes were up-regulated
265 and 83 genes were down-regulated (Table S7 and SI). The functional enrichment analysis revealed a significant
266 overrepresentation of genes coding for cuticular proteins and kinases/phosphatases among the up-regulated
267 genes, and of genes involved in immunity, intra- and extra-cellular trafficking or coding for
268 kinases/phosphatases among the down-regulated genes (Table 1, Table S7). The "*ccaat/enhancer binding*
269 *protein*" (*C/EBP*, AAEL002853) was the only transcription factor showing a differential transcription in larvae
270 following direct exposure (–1.6 fold). In insects, this transcription factor has been associated with the activation
271 of genes involved in various physiological functions, such as immune response and iron or protein
272 homeostasis.[47–49]

273 According to the functional enrichment analysis, several genes related to immune response were overrepresented
274 amongst the down-regulated genes, including five anti-microbial peptides (*CECN, CECE, DEFC, DEFD,*
275 *DEFA*), two clip-domain serine proteases, two prophenoloxidases (*PPO4, PPO6*), the C-type lectin *CTLGA5* and
276 one macroglobulin/complement (Figure 4, C, D; Table S7). Interestingly, C/EBP binding domains were detected
277 in the upstream region of defensins in *A. aegypti* and shown to be essential for their regulation together with
278 other antimicrobial peptides genes.[47]

279 The overrepresentation of genes involved in intra/extra-cellular trafficking was mainly due to the down-
280 regulation of all nine hexamerins annotated in the *A. aegypti* genome from -3.5 to -45.7 fold (Figure 4, C; Table
281 S7). Hexamerins play a crucial role as amino acid resources for imaginal metamorphosis in holometabolous
282 insects. Hexamerins are synthetized in the mosquito's fat body mainly during the 4$^{th}$ larval stage and proteolized
283 during the non-feeding nymphal stage, thus providing the amino acid resources needed for the development of
284 the future imago.[49,50] In *A. aegypti*, protein biosynthesis during 4$^{th}$ larval stage contributes to 70 to 88% of the
285 total protein quantity disposable for the nymphal metamorphosis.[33] The substantial down-regulation of
286 hexamerin transcription in F0 4$^{th}$ stage larvae exposed to ibuprofen should therefore affect the performance and
287 protein resources of exposed pupae and imagoes. However, this down-regulation did not result in an alteration in
288 the 15 free amino acid levels measured by GC-MS (Table S6) or the total cytosolic protein content (Figure S6) in
289 F0 imagoes. This demonstrates that the individual scale consequences of transcriptional changes should be
290 correlated to functional or phenotypic changes.

291 Some studies also suggested that hexamerins could be involved in innate immune response in mosquitoes and
292 other insects species.[51–53] The down-regulated genes involved in intra/extra-cellular trafficking also included two

293 transferrin and two ferritin coding genes (Figure 4, C; Table S7). These proteins, mainly synthesized in the fat
294 bodies and basically involved in iron transport, are also known to play a role in immune responses in insects.[54,55]
295 Interestingly, C/EBP binding sites have been shown to regulate hexamerins and ferritins in the *Aedes* species.[48,49]
296 Finally, four genes encoding odorant binding proteins, a large protein family found to be involved in immune
297 response[56] were also up-regulated in exposed larvae (Figure 4, D; Table S7).

298 In insect species, prostaglandins play a role in the immune response by mediating the induction of immune
299 genes, and eicosanoid biosynthesis inhibition in insects has been shown to disrupt the immune response.[57–60] The
300 down-regulation of immune response genes and hexamerins observed in F0 larvae (Figure 4, C-D) is thus
301 consistent with a disruption in prostaglandin synthesis, that might be induced by the inhibition of COX activity
302 (Figure 4, A). This is supported by the fact that the COX inhibitory activity of ibuprofen has been evidenced in
303 other invertebrate species, including the mosquito *Anopheles albimanus*.[25,61] As detailed above, several down-
304 regulated genes in F0 larvae are regulated by the C/EBP transcription factor. Interestingly, in mammal
305 macrophages the pro-inflammatory activity of prostaglandins has been shown to be mediated by the activation of
306 CREB, allowing it to bind to the C/EBP-β promoter and leading to an over-transcription of C/EBP-β expression
307 and of its target genes.[62] This suggests that the under-transcription of immune response genes and hexamerins
308 may be at least partially due to the down-regulation of C/EBP, through a disruption of prostaglandin synthesis in
309 exposed larvae (Figure 4 A, C). However, the influence of prostaglandins on C/EBP expression has never been
310 investigated in insect species, and this hypothesis needs to be confirmed.

311 **Molecular changes associated with direct ibuprofen exposure in F0 imagoes**

312 Only 40 DTG were detected in ibuprofen-exposed F0 females (Table S8 and SI). Such a small number of DTG
313 did not allow for the identification of specific changes in physiological pathways or functions. Moreover, based
314 on the metabolite concentrations measured, the global metabolic state of F0 females and males did not seem to
315 be affected by ibuprofen exposure (Figure S4, B, C; Table S8). Furthermore, exposure did not affect ecdysteroid
316 levels, nor the transcription dynamic of five key genes associated with ecdysteroid response and vitellogenesis
317 (Figure S5). Taken together these results suggest that the transcriptional changes observed in exposed F0 4$^{th}$
318 stage larvae have very limited consequences on imagoes, both at molecular and individual scales.

319 **Indirect impact of ibuprofen exposure on metabolic resources of in F1 progeny eggs**

320 The metabolic resources available for F1 progeny development were measured in 1 to 4 hour-old eggs,
321 corresponding to the very beginning of embryonic development and before the initiation of maternal-zygotic

322 transition.[63] The multivariate analysis of metabolic profiles using ACP revealed a clear segregation between the
323 metabolic profiles of progeny obtained from exposed individuals and the controls (Figure 5, A). An OPLS-DA
324 was performed to investigate the metabolites responsible for the separation of the two exposure groups. The
325 model fitted well (R2X= 0.44, R2Y= 0.72 – p=0.011 according to permutation test), and showed good
326 predictability (Q2Y= 0.66 - p= 0.007 according to permutation test), confirming the divergence of metabolic
327 profiles. The univariate approach using the Wilcoxon rank-sum test revealed that 20 metabolites were
328 significantly underrepresented from 12% to 81% in eggs obtained from ibuprofen-exposed parents (Figure 5, B).
329 These 20 metabolites are all characterized by correlation coefficient p-corr ≥ |0.5| in the OPLS-DA model,
330 confirming a good consistency between the univariate and multivariate statistical approaches. The eggs obtained
331 from ibuprofen-exposed individuals contained lower levels of amino acids (12 out of the 17 detected amino
332 acids), carbohydrates (maltose, sucrose), polyols (glycerol, arabitol, myoinositol and ribitol), phosphoric acid
333 and ornithine (Figure 5, B), implying that metabolic resource internalization in eggs is affected by ibuprofen
334 exposure. As embryonic development only relies on maternal nutrients, such changes in metabolic resources
335 may modify the physiology and phenotype of the resulting embryo and larvae.[64]

336 **Impact of parental ibuprofen exposure on the transcriptional state of young F1 larvae**

337 *Global transcriptional responses and functional analysis*. The transcriptome analysis of F1 1st stage larvae
338 obtained from ibuprofen-exposed parents revealed a broad change in transcriptional state compared to the
339 controls, with 1,566 DTGs (detailed in SI). The functional enrichment analysis of up-regulated genes revealed an
340 overrepresentation of transcripts involved in epigenetic marks and transcription regulation (Table 2), which
341 could explain the divergent transcription profiles observed between the progeny of control versus exposed
342 parents. Genes involved in signal transduction and cell/tissue structure were also enriched among the up-
343 regulated genes (Table 2). The 450 down-regulated genes revealed an enrichment of genes involved in protein
344 metabolism, lipid metabolism, mitochondrial metabolism, detoxification and formation of the cuticle (Table 2).
345 Interestingly, of the 66 genes involved in protein metabolism, 53 encoded for proteases (SI). The change in
346 metabolic resources available for embryonic development thus seems to define a distinct physiological state in
347 the progeny, reflected by a shift in F1 larvae transcriptional state, most likely driven by the differential
348 expression of numerous transcription factors and epigenetic mark regulators.

349 *Increase in ecdysone signalling.* 20E signalling is mediated by the heterodimer of nuclear receptor ecdysone
350 receptor (EcR)/ultraspiracle (USP). EcR-USP activation by 20E leads to the transcriptional activation of three

351 "early-response genes": *Br*, *E74* and *E75*. These early-response genes code for the transcription factors
352 responsible for the regulation of a large set of 20E target genes, so-called "early-late" and "late-response
353 genes".[65] A more detailed analysis of the DTGs associated with the enriched functional classes revealed the up-
354 regulation of several 20E response genes (Table S9, A), although *EcR* was not detected in our analysis and its
355 coreceptor *USP* was not affected. Three 20E signalling cofactors were significantly up-regulated in progeny
356 from exposed individuals (Figure 6): (1) "*trithorax-related*" (*trr*, AAEL005380), a histone methyltransferase
357 which is known to associate with the EcR/USP heterodimer in *D. melanogaster* and co-activates EcR signalling
358 activity by modifying the chromatin structure of ecdysone responsive promoters[66] (2) AAEL017391, the unique
359 ortholog (*D. melanogaster*, 37%) of *nejire*, a histone acetyltransferase which is necessary for the up-regulation
360 of ecdysone early-responsive genes upon ecdysone signal[67] and (3) "*cap-and-collar"* (*Cnc*, AAEL015467,
361 AAEL005077), which play a major role in *D. melanogaster* development and is crucial for the transcription of
362 20E early-response genes like *Br* and *E75*.[68–70] The activity of these three cofactors has been shown to play a
363 crucial role in the activation of 20E early-response genes.[66,67] The up-regulation of these genes suggests that
364 larvae obtained from ibuprofen-exposed parents display a transcriptional state favourable to ecdysone signalling
365 which could result in an overactivation of this pathway. This was confirmed by the over-transcription of two 20E
366 early-response genes: *Br* (AAEL008426) and *E75* (AAEL007397); four 20E early-late response genes: HR3
367 (AAEL009588, AAEL015043), HR4 (AAEL005850) and FTz-F1β (AAEL001304); and of two late-responsive
368 genes *npc1* (AAEL003325), AAEL005369, the unique ortholog of "*crooked legs*" (*D. melanogaster* 35%)[71]
369 (Figure 6). By associating the over-transcription of the transcription factor *Br* to the up-regulation of its direct
370 target gene (*npc1*),[65] this observation tends to validate the transcriptional observations from a functional point of
371 view. Apart from E74 which was not detected in our study, all the described early and early-late response genes
372 were up-regulated in the progeny of exposed parents. This tends to confirm an overactivation of the ecdysone
373 signalling pathway, as many steps in the ecdysone signalling hierarchy are up-regulated in our study.
374 The expression of enzymes involved in ecdysteroid biosynthesis is controlled by several transcription factors,
375 allowing the accurate regulation of the hormone dynamics controlling the development of insects. In particular,
376 the transcription factor "without children protein" (woc) up-regulates the expression of the gene coding for the
377 enzyme allowing the first step of steroïdogenesis, and Drifter has been shown to participate in the expression of
378 two other steroidogenic enzymes involved in ecdysteroid synthesis.[72,73] Moreover, in addition to its coactivating
379 role in ecdysone signalling, Cnc also plays a key role in the transcriptional activation of genes involved in
380 ecdysone synthesis in *D. melanogaster*.[70] Genes encoding the steroidogenic transcription factors *woc*

381 (AAEL004320), Drifter (AAEL003560) and *Cnc* were up-regulated in larvae obtained from exposed parents.
382 This up-regulation may contribute to the greater ecdysone signalling induced by parental exposure, although this
383 assumption is mitigated by the stable transcription level of key steroidogenic enzymes genes (Figure 6, Table
384 S9).
385 Ecdysone signalling plays a crucial role in moult timing regulation, having a major influence on the duration of
386 development of *A. aegypti*.[32] This timing regulation is notably due to the influence of 20E signalling on cell-
387 cycle regulation.[71] The transcriptional profile of larvae obtained from ibuprofen-exposed parents thus suggests
388 that increased ecdysone signalling causes the accelerated development observed in this progeny.

389 More broadly, the up-regulation of genes involved in "signal transduction" and "cell/tissue structure" functional
390 classes seems to fit with the greater developmental rate observed in the progeny of exposed individuals (Table
391 2). Indeed, Mensch *et al*. demonstrated that an increase in the developmental rate of *Drosophila melanogaster*
392 was associated with an increased expression of genes involved in these functions.[74]

393 *Increase in stress response transcriptional potential.* Up-regulated genes associated with "transcription
394 regulation" include the *heat-shock factor* (*hsf*, AAEL010319), a transcription factor well described as being
395 responsible for controlling stress-response gene expression.[75] In *D. melanogaster*, the regulation of HSF
396 transcriptional activity under stress conditions is well described. The expression of heat shock proteins (HSP)
397 under stress conditions requires the recruitment by HSF of the P-TEFb complex (Cdk9/cyclin T) to the upstream
398 region of *hsp* genes. Activation of *hsp70* transcription on stress exposure also requires chromatin re-modelling
399 due to FACT complex and histone chaperone Spt6.[75] Two of these genes essential for heat-shock protein (HSPs)
400 production on stress exposure such as cyclin t (AAEL004839) and the ortholog of *spt6* (*D. melanogaster,* 55%)
401 annotated as "suppressor of ty" (AAEL006956) on the *A. aegypti* genome were also up-regulated (Table S10).
402 Interestingly, the up-regulation of these genes specifically involved in *hsp* gene expression was associated with a
403 basal over-transcription of 7 genes encoding HSPs or associated proteins (Figure 6, Table S10). Higher basal
404 levels of HSP mRNA and the up-regulation of the genes necessary for HSPs production under stress exposure
405 may result in a more efficient response to stress in the progeny of exposed parents. Interestingly, as well as
406 regulating ecdysone signalling, the transcription factor CnC is also responsible for the xenobiotic-stress response
407 in *D. melanogaster*, and the activation of its signalling confers improved tolerance to insecticides.[76]
408 Several genes specifically involved in the nutritional stress response were also up-regulated in F1 larvae obtained
409 from exposed parents: (1) the histone deacetylase coded by *sir2* (AAEL006655), involved in the mobilisation of
410 fat stores and the regulation of lipid metabolism in response to nutritional stress, which is crucial for starvation-

411 survival in *D. melanogaster*[77]; (2) the unique ortholog (*D. melanogaster*, 47%) of HDAC4 deacetylase
412 AAEL002528 and *lipin* (AAEL005175) (Figure 6, Table S10) which are also involved in the regulation of lipid
413 metabolism in response to food deprivation in *D. melanogaster.*[78,79] Lipin notably up-regulates genes involved in
414 fatty-acid oxidation under starvation conditions, allowing the metabolism to adapt to the decrease in energy
415 resources.[79] These three genes are up-regulated under starvation conditions in *D. melanogaster*, and Sir2
416 overexpression led to an increased tolerance to starvation.[80] Interestingly, in *D. melanogaster* imagoes starvation
417 induces the up-regulation of the genes involved in growth, maintenance, and protein synthesis while peptidases,
418 trafficking, detoxification and immunity proteins are down-regulated.[81] This starvation-induced transcriptional
419 profile is strongly consistent with the up-regulation of genes involved in cell/tissue structure and the specific
420 down-regulation of genes involved in detoxification and proteases observed in our F1 larvae after parental
421 exposure (Table 2).
422 Taken together these results suggest that the transcriptional state of the progeny of exposed parents could confer
423 an improved response potential to stressful events, particularly nutritional stress, and may explain the better
424 tolerance to starvation observed after parental exposure to ibuprofen.

425 *Increase in cell signalling potential*. A detailed analysis of the up-regulated genes associated with the "signal
426 transduction" functional class revealed their role in several signalling pathways, including insulin, MAPK, and
427 TOR (Figure 6, Table S11). Insulin signal mainly regulates PI3K/AKT/PKB pathways, leading to the regulation
428 of the TOR and ERK/MAPK pathways.[82] Several genes belonging to the insulin signalling pathway were up-
429 regulated in the progeny of exposed individuals, including the three genes coding for PI3K (AAEL013596,
430 AAEL002903 and AAEL000386), the regulatory proteins *fak* (AAEL004666) and *pp2a* (AAEL014042) and the
431 effectors *tsc2* (AAEL007712) and *rac* (AAEL008823) (Figure 6, Table S11). Genes associated to the TOR
432 pathway were also up-regulated including the three regulators *Rheb* (AAEL008179), *GPRFZ2* (AAEL006778)
433 and the unique ortholog (*D. melanogaster*. 52%) of *happyhour* (AAEL017338), and the TOR substrate S6K
434 (AAEL018120), albeit *tor* (AAEL000693) transcription level was not affected by parental exposure. Regulation
435 of ERK/MAPK pathway also appeared affected by parental exposure to ibuprofen, as *shc* (AAEL008739), *sos*
436 (AAEL012071), *ras* (AAEL012071), *erk1/2* (AAEL007958, AAEL013939) and *mnk* (AAEL004704) were up-
437 regulated (Figure 6, Table S11).

438 Interestingly, multiple studies have demonstrated the role of insulin and TOR signalling pathways on 20E
439 signalling regulation in insects.[83–85] Overexpression of the activated form of Ras in D. melanogaster leads to

440 precocious pupation through the activation of the ecdysone signalling pathway, evidencing the role of insulin
441 signalling pathway activation state in determining development time.[85] It was suggested that this action of Ras
442 was due to the transcriptional activation of 20E synthesis genes by CnC, by favouring the interaction of CnC
443 with chromatin and its transcriptional activity.[86] The TOR pathway regulates the expression of ecdysteroid
444 synthesis related genes and 20E-response genes in response to amino acid levels, depending on the
445 transmembrane receptor Frizzeled2 in *A. aegypti*.[87,88] TOR pathway also regulates cell and body growth in
446 response to the cellular nutritional and energetic state through a well-described mechanism in *D.*
447 *melanogaster*.[89,90] Interestingly, the genes encoding the TOR regulator Frizzeled 2 (*gprfz2*, AAEL006778) and
448 several effectors of the TOR pathway specifically involved in the nutritional stress response, *pp2a* and *14-3-3*
449 (AAEL011116), *lipin* and the unique ortholog (*D. melanogaster*, 33%) of *reptor* (AAEL009171), were up-
450 regulated in the progeny of exposed parents (Figure 6, Table S11).[91] Insulin and TOR pathways thus participate
451 in insects, in the regulation of growth and development timing according to the availability of nutritional
452 resources through the regulation of the ecdysone signalling pathway and regulates the implementation of the
453 nutritional stress response.[91–93]

454 Given the vital role of these pathways in development regulation and specific stress-response implementation,
455 overrepresentation of these general pathways (Insulin, MAPK and TOR) and specific effectors in F1 larvae
456 transcriptome suggests that their transcriptional state increases their potential for integrating environmental cues,
457 especially nutritional ones, allowing the potentiation of appropriate responses.

458 **Origin of the intergenerational consequences of ibuprofen exposure**

459 Overall, our approach revealed a larval transcriptional response that could be consistent with the
460 pharmacological mode of action of ibuprofen, with moderate molecular and phenotypic consequences at
461 imaginal stages. However, parental exposure led to lower levels of several metabolites in eggs, including amino
462 acids. This change in the metabolic resources available for embryonic development results in a distinct
463 physiological state in the progeny, reflected by a shift in the F1 larvae transcriptional state and affecting their
464 phenotype. The transcriptional state of the progeny displays an overactivation of the ecdysone signalling
465 pathway resulting in the acceleration of larval development and seems to constitute preparation for a rapid and
466 efficient response to a stressful environment, explaining the larvae's better tolerance of starvation.

467 Thus, changes in progeny phenotype are likely to be initiated by a differential internalization of resources in eggs
468 by exposed parents. In *A. aegypti*, resource internalization in eggs occurs in females during the vitellogenic

phase, and the expression of genes necessary to the transport and internalization of metabolic resources are tightly regulated by ecdysone.[94] However, our approach failed to demonstrate any changes at molecular scale in exposed imagoes that could explain such changes in reproductive investment, especially concerning female hormonal state and metabolic resources. Assuming that F0 imagoes are no longer in direct contact with ibuprofen, the intergenerational impact could result from a delayed consequence of larval exposure, such as a modification in the expression potential of the specific genes involved in resource internalization in females, in response to 20E signalling, without influencing 20E synthesis nor the transcriptomic profile at the pre-vitellogenic stage. These types of delayed impacts are notably suspected of being due to changes in the epigenetic regulation of transcription.[95] This must involve a differential expression of one or several genes involved in metabolic resource internalization during the egg maturation process. An untargeted transcriptomic approach during the vitellogenesis may have revealed such transcriptional changes on genes other than the best described vitellogenic genes we specifically focused on. This clearly illustrates the limit of targeted approaches for deciphering intergenerational mechanisms, since the factors involved in egg maturation and progeny production are highly multifactorial.

## Towards a better understanding of the intergenerational impact of pollutants

Our study investigated the consequences of direct and parental chronic exposure to ibuprofen in the model insect *A. aegypti*. It illustrates the interest of a system approach in mechanistic studies, as it not only helps to deepen our understanding of the molecular basis of observed phenotypic variations, but also provides new insights into the consequences of pollutant exposure, including in the context of stress-on-stress. Such studies investigating the impacts of chronic exposure to environmentally relevant concentrations of pollutants were scarce until recent years. However, populations are often chronically exposed to micropollutants in the environment. Our work particularly emphasizes the importance of temporal scale in ecotoxicity studies and shows that considering the entire life cycle of organisms, including that of their progeny, is of major relevance to fully understand the impacts of chronic pollutant exposure and the associated mode of action. A precise understanding of the upstream molecular events leading to inter- and transgenerational impacts of chemicals is of particular interest as it is key to improving the prediction of transgenerational adverse outcomes, and to enhancing the ecological relevance of ecotoxicological risk assessment.[95] Interestingly, the consequences of parental ibuprofen exposure on F1 larvae may seem to be beneficial in terms of the survival potential of progeny larvae, raising the question of the specificity of the observed effects: are the changes occurring in F1 progeny due to a specific alteration induced by ibuprofen or do they reflect a modified reproductive strategy in response to a potentially changing

environment? This adaptive aspect of the inter- and transgenerational impacts of pollutants should be carefully considered in multigenerational ecotoxicological studies, as both reproductive plasticity and specific disruption elicited by contaminants are susceptible to regulate the phenotype in the progeny.[91]

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**SUPPORTING INFORMATION:**

The Supporting Information is available free of charge on the ACS Publications website at DOI:

Detailed Materials and Methods, Table S1−S11, Figure S1−S6 (PDF)

Detailed transcriptomic data (Table sheet)

**DATA AVAILABILITY STATEMENT:**

The RNA-seq sequence data from this study have been deposited at the European Nucleotide Archive (ENA; http:// www.ebi.ac.uk/ena) under the accession numbers PRJEB23849 and PRJEB23850.

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## FIGURES CAPTIONS

**Figure 1: Effect of ibuprofen exposure on *Aedes aegypti* imago lifespan. A**: Lifespan of virgin females exposed to 1µg/L ibuprofen (pink curves, n = 32) or solvent control (black curve, n = 34). **B**: Lifespan of primiparous females exposed to 1µg/L ibuprofen (pink curves, n = 52) or solvent control (black curve, n = 54). **C**: Lifespan of males exposed to 1µg/L ibuprofen (pink curves, n = 59) or solvent control (black curve, n = 67). P-values are given according to a Kaplan-Meyer survival test.

**Figure 2: Effect of ibuprofen parental exposure on progeny survival under starvation conditions.** First stage larvae from the progeny of populations exposed to 1µg/L ibuprofen (pink curves, n = 238) or solvent control (black curve, n = 237) were raised in media with no nutritional resources. P-value is given according to a Kaplan-Meyer survival test.

**Figure 3: Differential gene transcription in *Aedes aegypti* after direct or parental ibuprofen exposure.** Three pools of 4$^{th}$ stage larvae (**L4F0**, n=35) and female imagoes (**FemF0**, n=30) exposed to 1µg/L ibuprofen or solvent control were respectively collected 40h after moult and 72h after emergence. Three pools of F1 1$^{st}$ stage larvae from each parental exposure condition (**L1F1**, n=300) were collected 10 hours after hatching. Gene transcription was characterized by RNAseq and statistically significant differential transcription following

770 ibuprofen direct or parental exposure compared to solvent control individuals or progeny was assessed (|FC|>1,2;
771 q-value<0.05). **A**: Comparison of detected, up-regulated and down-regulated genes. **B**: Diagram showing the
772 number of differentially transcribed genes shared by the three developmental stages studied. **C**: Comparison of
773 transcription fold-changes associated to differentially transcribed genes; ***: FDR-adjusted p-value<0.001.

774 **Figure 4: Transcriptomic response of *Aedes aegypti* 4th stage larvae (F0) chronically exposed to 1µg/L**
775 **ibuprofen**. **A**: Putative mechanism involved in transcriptomic perturbation induced by ibuprofen exposure. This
776 mechanism is based on knowledge of the pharmacological mode of action of ibuprofen in vertebrates and the
777 consequences of prostaglandin pathway disruption in insects. Under the control conditions, arachidonic acid
778 (AA) is released from membrane phospholipids and diacylglycerol by phospholipase A2 (**$PLA_2$**) and
779 phospholipase C (**PLC**). AA is converted in prostaglandin $H_2$ ($PGH_2$) by cyclooxygenases 1&2 (**COX1**,
780 **COX2**).[20] Prostaglandins regulate several cellular processes including the induction of immune genes[58,59]
781 through the regulation of the transcription factor CCAAT/enhancer binding protein (**C/EBP**) expression[62] *(1)* or
782 unknown mechanisms *(2)*. By inhibiting COX activities, ibuprofen reduces $PGH_2$ synthesis thus reducing the
783 expression of immune related genes and C/EBP target genes. **B**, **C**, **D**: The blue/orange colour-scale indicates
784 transcription level variations in exposed larvae compared to control. **B**: Impact of ibuprofen exposure on
785 transcription factors transcription rate. **C**: Impact of ibuprofen exposure on potential C/EBP target genes
786 transcription rate. **D**: Impact of ibuprofen exposure on other immunity related genes transcription rate.

787 **Figure 5: Metabolome of *Aedes aegypti* F1 eggs spawned by control or ibuprofen exposed F0 individuals.**
788 **A**: Principal component analysis (PCA) performed with the quantification value of the 50 detected metabolites
789 from 6 samples of 100 eggs for each condition. **B**: Percent change in the concentrations of metabolites
790 significantly affected by parental exposure to ibuprofen according to a Wilcoxon rank-sum test. Bars are
791 coloured according to the correlation score of the metabolites to OPLS-DA (R2X: 0.44, R2Y: 0.72, Q2: 0.66). *:
792 FDR-adjusted p-value<0.05. **: FDR-adjusted p-value<0.01. *a*: amino acids. *b*: carbohydrates. *c*: polyols. *d*:
793 intermediate metabolites.

794 **Figure 6: Effect of chronic parental ibuprofen exposure on F1 progeny gene transcription**. The blue/orange
795 colour-scale indicates transcription level variations in exposed larvae compared to control. Orthlog (unique) and
796 percent similarity with *Drosophila melanogaster* genes are given between brackets. **A**: Signalling pathway genes
797 differential expression. Each gene found differentially transcribed has been assigned to specific signalling
798 pathway according to the Kyoto Encyclopedia of Genes and Genomes (KEGG) (*D.mel*) and bibliography.[82,91]

799 Plain arrows indicate direct protein interaction and dashed arrows indicate biological process regulation.
800 ERK/MAPK signalling pathway – *SHC: Src homology 2 domain containing. GRB2: growth factor receptor-*
801 *bound protein. SOS: son of sevenless* (AAEL012071). *MEK: mitogen activated protein kinase kinase. ERK:*
802 *mitogen-activated protein kinase* (AAEL007958; AAEL013939). *MNK: MAP kinase interacting*
803 *serine/threonine kinase.* Insulin signalling pathway – *INSR: insulin receptor. IRS1: insulin receptor substrate 1.*
804 *PI3K: phosphoinositide-inositol 3 kinase. PDK1: 3-phosphoinositide dependent protein kinase. Akt/Rac: Rac*
805 *serine/threonine kinase. TSC1: Tuberous sclerosis complex 1. TSC2: Tuberous sclerosis complex 2. FAK: focal*
806 *adhesion kinase.* TOR signalling pathway – *hppy: happyhour. Rheb: Ras homolog enriched in brain. GPRFZ2:*
807 *Fizzeled 2. TOR: target of rapamycin. S6K: ribosomal protein S6 kinase. 4EBP: eukaryotic translation initiation*
808 *factor 4E binding protein. REPTOR-BP: reptor binding protein.* **B**: Genes involved in ecdysone signalling. The
809 arrow indicates direct transcriptional regulation. *CnC: Cap'n'collar. woc: without children. setd: set domain*
810 *protein. Br: broad-complex. E75: ecdysone inducible protein E75. HR3: hormone receptor 3. HR4: hormone*
811 *receptor 4. FTz-f1β: ftz transcription factor 1.* **C**: Genes involved in stress response. The arrow indicates direct
812 transcriptional regulation. *HSF: heat shock factor. HSPs: heat shock proteins. Sir2: sirtuin 2. HDAC4: histone*
813 *deacelyase. PP2A: protein phosphatase 2A.*

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815

816 ***Table 1 – Functional class enrichment study based on genes differentially transcribed between solvent***
817 ***control and Ibuprofen exposed 4$^{th}$ stage larvae. The 134 genes significantly differentially transcribed in***
818 ***exposed larvae were used for functional class enrichment according to a Fisher's exact test, corrected for***
819 ***multiple comparison*** *using Benjamini-Hochberg method.*

**Up-regulated genes (51)**

| **Functional class** | **Enrichment** | ***p-value*** | **Implicated genes** |
|---|---|---|---|
| Kinases and phosphatases | x 4.45 | 3.33E-02 | 3 |
| Cuticle | x 15.83 | 2.30E-05 | 5 |

**Down-regulated genes (83)**

| **Functional class** | **Enrichment** | ***p-value*** | **Implicated genes** |
|---|---|---|---|
| Immunity | x 6.16 | 4.29E-06 | 11 |
| Kinases and phosphatases | x 4.56 | 5.83E-03 | 5 |
| Intra/extracellular trafficking - Chaperones | x 3.94 | 7.16E-06 | 17 |
| Others | x 1.65 | 1.14E-03 | 6 |

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821

822 ***Table 2 – Functional class enrichment study based on genes differentially transcribed between solvent***
823 ***control and Ibuprofen exposed progeny (1st stage larvae). The 1566 genes significantly differentially***
824 ***transcribed in exposed larvae were used for functional class enrichment according to a Fisher's exact test,***
825 ***corrected for multiple comparison*** *using Benjamini-Hochberg method.*

**Up-regulated genes (1116)**

| **Functional class** | **Enrichment** | ***p-value*** | **Implicated genes** |
|---|---|---|---|
| Epigenetic marks regulation | x 3.02 | 2.84E-04 | 15 |
| Transcription regulation | x 1.70 | 2.49E-06 | 65 |
| Signal transduction | x 2.20 | 2.20E-13 | 99 |
| Structure | x 1.68 | 1.11E-02 | 127 |

**Dow-regulated genes (450)**

| **Functional class** | **Enrichment** | ***p-value*** | **Implicated genes** |
|---|---|---|---|
| Lipid metabolism | x 1.64 | 2.93E-02 | 22 |
| Protein metabolism | x 1.54 | 8.37E-04 | 66 |
| Detoxification | x 2.95 | 2.73E-08 | 35 |
| Mitochondrial metabolism | x 2.03 | 2.14E-02 | 11 |
| Cuticle | x 4.95 | 3.19E-07 | 15 |
| Others | x 1.65 | 4.34E-02 | 10 |

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834 **Figure 1**

835

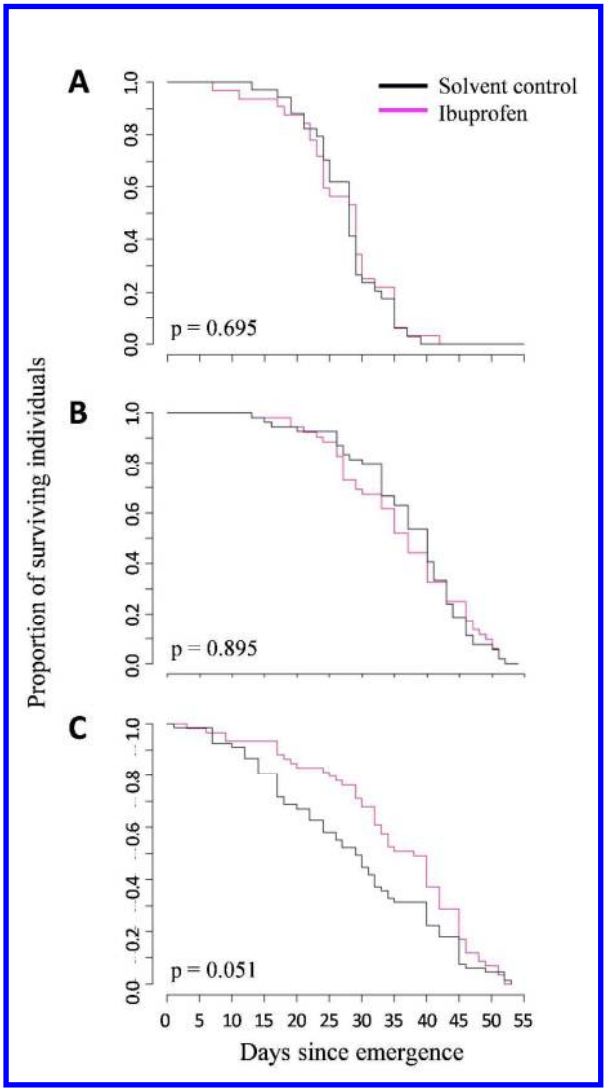


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**Figure 2**

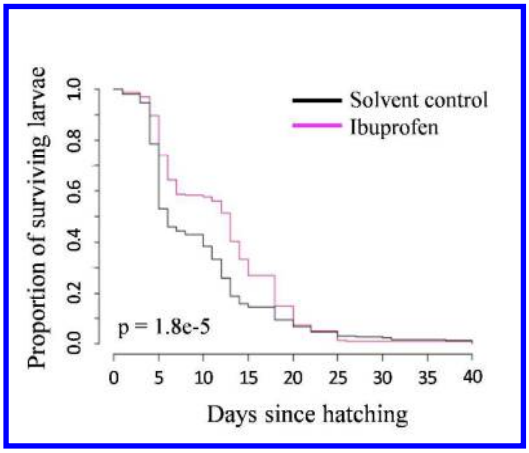

Proportion of surviving larvae
0.0
0.2
0.4
0.6
0.8
1.0
Solvent control
Ibuprofen
p = 1.8e-5
0
5
10
15
20
25
30
35
40
Days since hatching

**Figure 3**

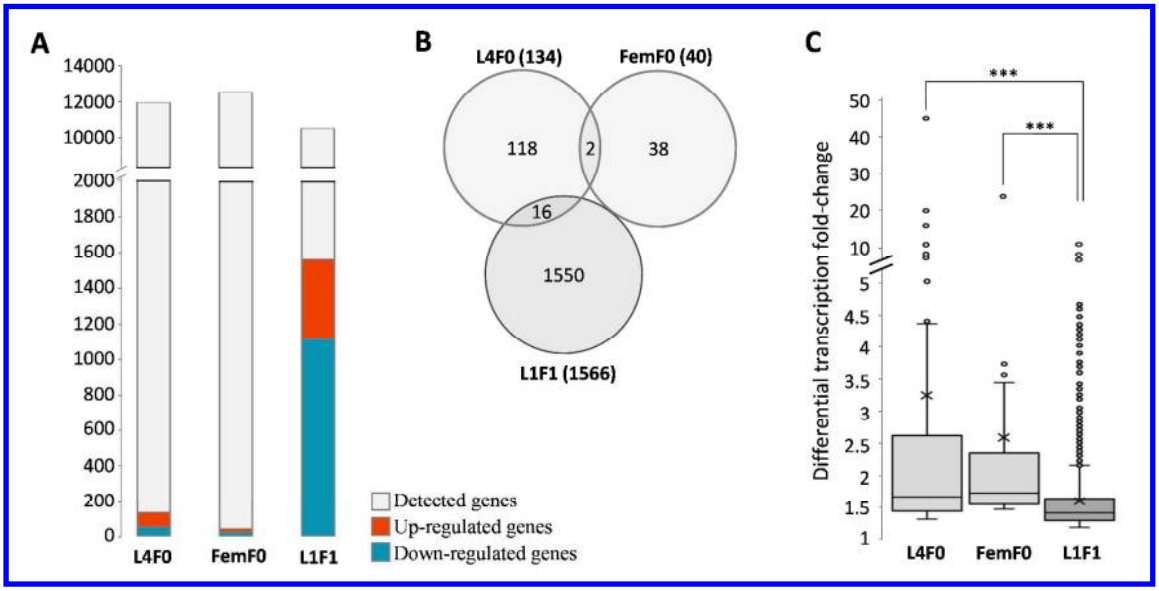

A
14000
12000
10000
2000
1800
1600
1400
1200
1000
800
600
400
200
0
L4F0
FemF0
L1F1
Detected genes
Up-regulated genes
Down-regulated genes
B
L4F0 (134)
FemF0 (40)
118
2
38
16
1550
L1F1 (1566)
C
Differential transcription fold-change
50
40
30
20
10
5
4.5
4
3.5
3
2.5
2
1.5
1
***
***
L4F0
FemF0
L1F1

**Figure 4**

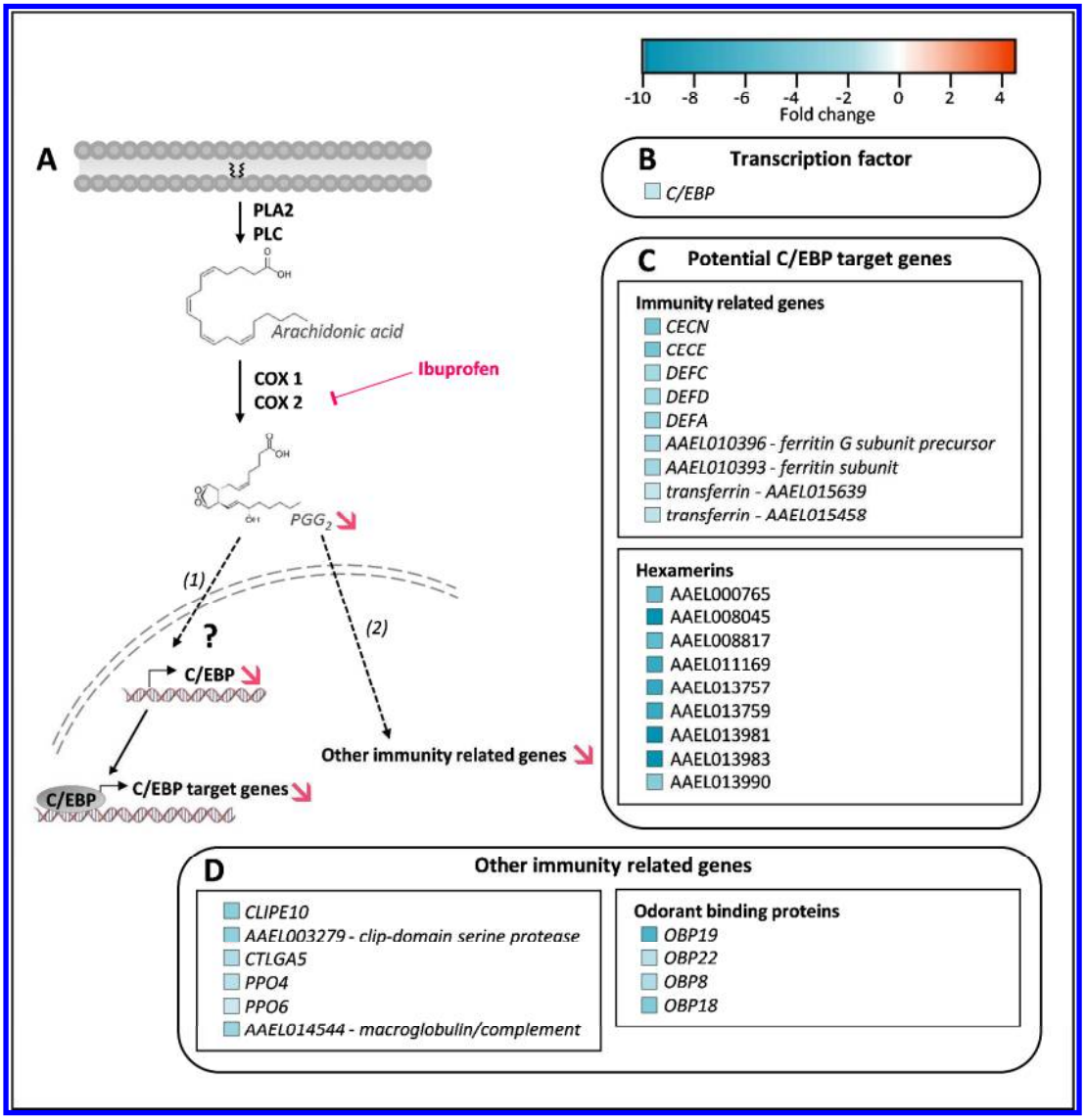

-10 -8 -6 -4 -2 0 2 4
Fold change
A
PLA2
PLC
Arachidonic acid
COX 1
COX 2
Ibuprofen
PGG2
(1)
(2)
?
C/EBP
C/EBP target genes
Other immunity related genes
B
Transcription factor
C/EBP
C
Potential C/EBP target genes
Immunity related genes
CECN
CECE
DEFC
DEFD
DEFA
AAEL010396 - ferritin G subunit precursor
AAEL010393 - ferritin subunit
transferrin - AAEL015639
transferrin - AAEL015458
Hexamerins
AAEL000765
AAEL008045
AAEL008817
AAEL011169
AAEL013757
AAEL013759
AAEL013981
AAEL013983
AAEL013990
D
Other immunity related genes
CLIPE10
AAEL003279 - clip-domain serine protease
CTLGA5
PPO4
PPO6
AAEL014544 - macroglobulin/complement
Odorant binding proteins
OBP19
OBP22
OBP8
OBP18

**Figure 5**

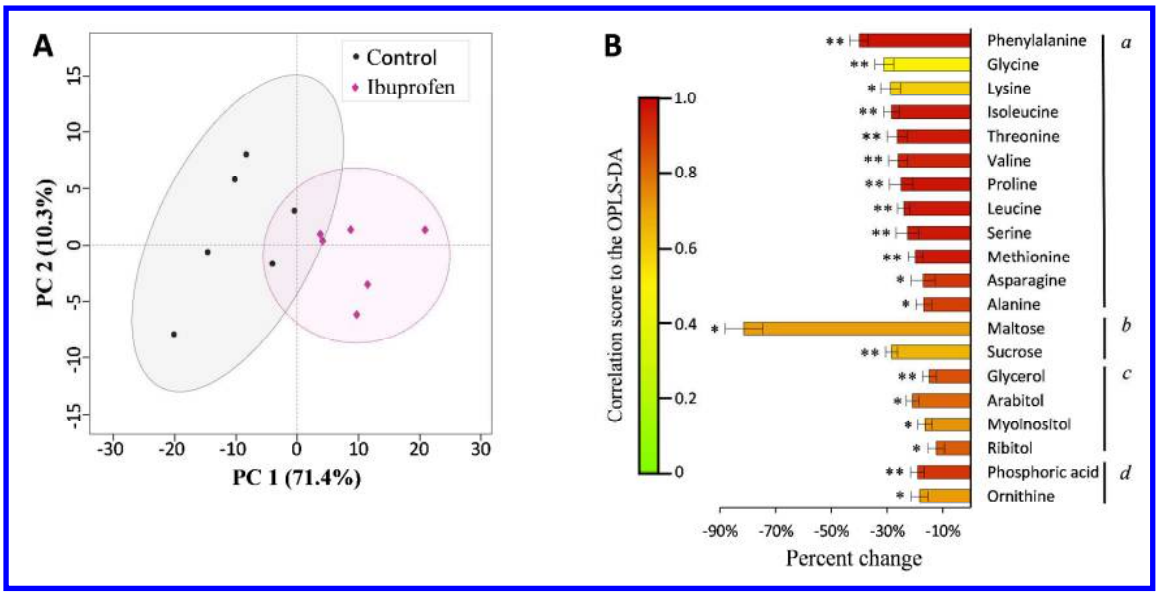
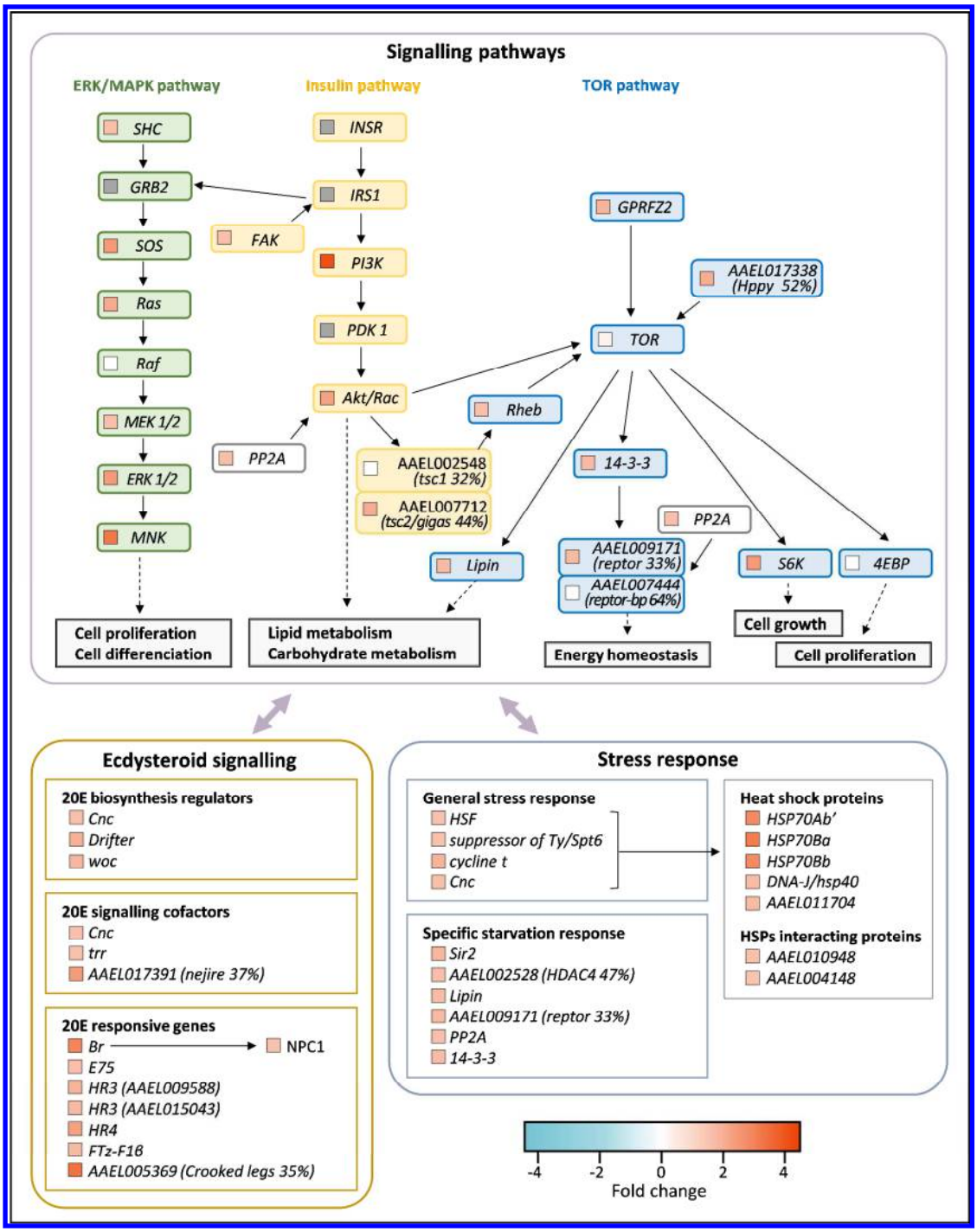
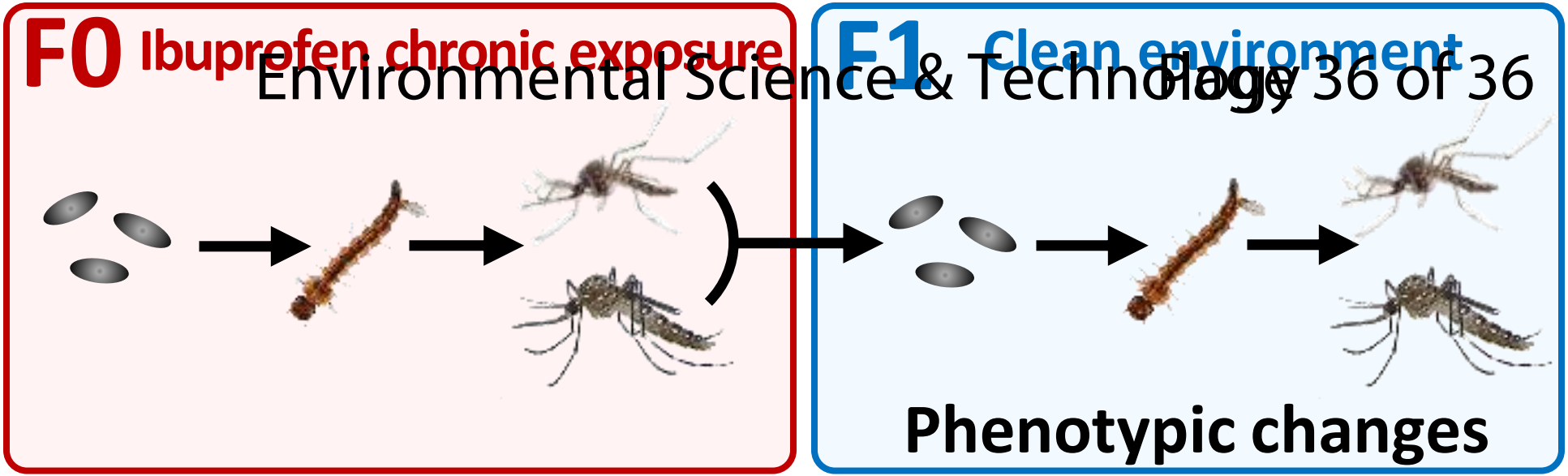

A
Control
Ibuprofen
PC 2 (10.3%)
PC 1 (71.4%)
B
Correlation score to the OPLS-DA
Phenylalanine
Glycine
Lysine
Isoleucine
Threonine
Valine
Proline
Leucine
Serine
Methionine
Asparagine
Alanine
Maltose
Sucrose
Glycerol
Arabitol
Myoinositol
Ribitol
Phosphoric acid
Ornithine
a
b
c
d
Percent change

**Figure 6**


Signalling pathways
ERK/MAPK pathway
Insulin pathway
TOR pathway
SHC
GRB2
SOS
Ras
Raf
MEK 1/2
ERK 1/2
MNK
INSR
IRS1
FAK
PI3K
PDK 1
Akt/Rac
PP2A
AAEL002548 (tsc1 32%)
AAEL007712 (tsc2/gigas 44%)
Rheb
GPRFZ2
AAEL017338 (Hppy 52%)
TOR
14-3-3
PP2A
AAEL009171 (reptor 33%)
AAEL007444 (reptor-bp 64%)
Lipin
S6K
4EBP
Cell proliferation
Cell differenciation
Lipid metabolism
Carbohydrate metabolism
Energy homeostasis
Cell growth
Cell proliferation
Ecdysteroid signalling
20E biosynthesis regulators
Cnc
Drifter
woc
20E signalling cofactors
Cnc
trr
AAEL017391 (nejire 37%)
20E responsive genes
Br
NPC1
E75
HR3 (AAEL009588)
HR3 (AAEL015043)
HR4
FTz-F1β
AAEL005369 (Crooked legs 35%)
Stress response
General stress response
HSF
suppressor of Ty/Spt6
cycline t
Cnc
Heat shock proteins
HSP70Ab'
HSP70Ba
HSP70Bb
DNA-J/hsp40
AAEL011704
HSPs interacting proteins
AAEL010948
AAEL004148
Specific starvation response
Sir2
AAEL002528 (HDAC4 47%)
Lipin
AAEL009171 (reptor 33%)
PP2A
14-3-3
-4
-2
0
2
4
Fold change




Transcriptomic
Metabolomic
Hormone levels

ACS Paragon Plus Environment

**Toxicity pathways**

**Intergenerational MOA**