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Zhang, Chao; Jones, Martin; Govaert, Lynn; Viant, Mark; De Meester, Luc; Stoks, Robby

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Resurrecting the metabolome: Rapid evolution magnifies the metabolomic plasticity to predation in a natural *Daphnia* population

Running title: Evolution magnifies metabolomic plasticity

Chao Zhang*[1,2], Martin Jones[3], Lynn Govaert[4], Mark Viant[3], Luc De Meester[5], Robby Stoks[1]

[1] Environmental Research Institute, Shandong University, Qingdao, China

[2] Evolutionary Stress Ecology and Ecotoxicology, KU Leuven, Leuven, Belgium

[3] School of Biosciences, University of Birmingham, Birmingham, UK

[4] Department of Aquatic Ecology, Swiss Federal Institute of Aquatic Science and Technology, Dübendorf, Switzerland

[5] Laboratory of Aquatic Ecology, Evolution and Conservation, KU Leuven, Leuven, Belgium

Authors' emails:

Chao Zhang (chaozhang@sdu.edu.cn; chao.zhang@kuleuven.be)

Martin Jones (m.r.jones.1@bham.ac.uk)

Lynn Govaert (lynn.govaert@eawag.ch)

Mark Viant (m.viant@bham.ac.uk)

Luc De Meester (luc.demeester@kuleuven.be)

Robby Stoks (robby.stoks@kuleuven.be)

* **Corresponding author:** Chao Zhang

chaozhang@sdu.edu.cn; Phone: +86-0532-58631989; Fax: +86-0532-58631981

Mailing address: Environmental Research Institute, Coastal highway 72, Qingdao, 266237, China

Abstract

Populations rely on already present plastic responses (ancestral plasticity) and evolution (including both evolution of mean trait values, constitutive evolution, and evolution of plasticity) to adapt to novel environmental conditions. Because of the lack of evidence from natural populations, controversy remains regarding the interplay between ancestral plasticity and rapid evolution in driving responses to new stressors. We addressed this topic at the level of the metabolome utilizing a resurrected natural population of the water flea *Daphnia magna* that underwent a human-caused increase followed by a reduction in predation pressure within ~16 years. Predation risk induced plastic changes in the metabolome which were mainly related to shifts in amino acid and sugar metabolism, suggesting predation risk affected protein and sugar utilization to increase energy supply. Both the constitutive and plastic components of the metabolic profiles showed rapid, likely adaptive evolution whereby ancestral plasticity and evolution contributed nearly equally to the total changes of the metabolomes. The subpopulation that experienced the strongest fish predation pressure and showed the strongest phenotypic response, also showed the strongest metabolomic response to fish kairomones, both in terms of the number of responsive metabolites and in the amplitude of the multivariate metabolomic reaction norm. More importantly, the metabolites with higher ancestral plasticity showed stronger evolution of plasticity when predation pressure increased, while this pattern reversed when predation pressure relaxed. Our results therefore highlight that the evolution in response to a novel pressure in a natural population magnified the metabolomic plasticity to this stressor.

Key words: adaptation; ancestral plasticity; crustaceans; metabolomics; rapid evolution of metabolic profiles; resurrection ecology

Introduction

Natural populations are facing rapid and strong environmental changes that ask for prompt responses to avoid local extinction. To realize these rapid responses to new environmental conditions, populations may rely not only on already present plastic responses (i.e. ancestral plasticity) but also on rapid evolutionary changes, whereby the latter can involve both evolution of mean trait values (i.e., constitutive evolution) and evolution of plasticity (Fox, Donelson, Schunter, Ravasi, & Gaitán-Espitia, 2019; Ghalambor, McKay, Carroll, & Reznick, 2007; Russell Lande, 2015). Against this background, important outstanding questions in evolutionary biology are what the relative contributions of the plastic and evolutionary responses are and how these relate to each other in determining rates of rapid adaptation (Fox et al., 2019; Ghalambor et al., 2015; Levis & Pfennig, 2016; López-Maury, Marguerat, & Bähler, 2008). There is still a debate going on regarding whether plasticity helps or hinders evolution. On the one hand, there is the view that ancestral plasticity may facilitate adaptive evolution, hence ancestral plasticity and the subsequent evolutionary response may covary positively (Espinosa-Soto, Martin, & Wagner, 2011; Scoville & Pfrender, 2010). On the other hand, the opposite has been hypothesized: that plasticity may slow down evolution by shielding traits from natural selection (Huey, Hertz, & Sinervo, 2003; Price, Qvarnström, & Irwin, 2003), resulting in negative covariation patterns between both. A major reason for why this controversy remains is the lack of compelling evidence from natural populations (Levis & Pfennig, 2016).

Because typically only the resulting phenotype after the joint action of plasticity and evolution can be studied, quantifying the ancestral plasticity is a key challenge when studying the interplay of plasticity and evolution in natural populations (Levis & Pfennig, 2016). Resurrection ecology offers a powerful solution when combined with common-garden

experiments where both the ancestral and derived genotypes of the same natural population that underwent a change in environmental conditions are reared in the absence and in the presence of the new selective agent. This combined retrospective approach allows reconstruction of microevolution of both trait means and their plasticity (Franks, Sim, & Weis, 2007; Weider, Jeyasingh, & Frisch, 2018). Resurrection ecology studies have been successfully applied to document evolutionary responses of natural populations to environmental stressors both at the levels of the phenotype (Hairston et al., 1999) and gene expression (Orsini, Spanier, & De Meester, 2012).

One way to advance insights in the interplay of ancestral plasticity and evolution in driving rapid trait adaptation is to focus on the underpinning molecular mechanisms (Fox et al., 2019). Such studies are very rare and limited to changes in gene expression (Ghalambor et al., 2015). Ignored so far in this context are responses in the metabolomes, the set of low-molecular-weight metabolites within organisms that is critical for unravelling the biochemical underpinnings of stress responses (Viant, Kurland, Jones, & Dunn, 2017). Metabolomic data can better reflect the activities at a functional level as metabolites are a downstream result of gene expression (Putri et al., 2013). Transcriptomic and proteomic data reveal the set of gene products being produced in the cell, data that represents one aspect of cellular function. Conversely, metabolic profiling can give an instantaneous snapshot of the physiology of that cell, and thus, metabolomics provides a direct "functional readout of the physiological state" of an organism (Hollywood, Brison, & Goodacre, 2006). Notably, metabolomics is also best suited to directly pick up any changes in free sugars which are expected under predation risk (meta-analysis of Rinehart & Hawlena, 2020), changes that other omics approaches (especially proteomics) would not be able to (directly) detect. Recently, metabolomics has been successfully applied to understand the plastic response to a variety of environmental

98 stressors (e.g., global warming, ocean acidification) (Calosi et al., 2017; Garreta-Lara,
99 Campos, Barata, Lacorte, & Tauler, 2018; Mayor, Sommer, Cook, & Viant, 2015). We,
100 however, lack information on the rapid evolution and its interplay with plasticity of
101 metabolomic profiles in natural populations, which may provide unique insights in the
102 interplay of ancestral plasticity and evolution at the molecular level.

103 Combining metabolomics with resurrection ecology and common-garden
104 experiments, we tested the plastic and evolutionary responses of the metabolome to predation
105 risk in a natural population of the water flea *Daphnia magna*. Predation is a potent selective
106 pressure in natural populations. There is widespread evidence that predators may generate not
107 only plastic but also rapid evolutionary responses in a wide array of phenotypic traits in prey
108 populations including life history (Benard, 2004; Reznick, Shaw, Rodd, & Shaw, 1997),
109 physiology (Hawlena & Schmitz, 2010), morphology (Eklöv & Svanbäck, 2017), and
110 behaviour (Schoener, Losos, Kolbe, Lapiedra, & Leal, 2018). Yet, the metabolomic responses
111 of prey (including both plastic and evolutionary responses) to predation remain unexplored.
112 We capitalized on a unique *D. magna* population that experienced strong and opposing
113 transitions in fish predation pressure through a period of ~16 years. First, there was a period
114 with no fish predation (i.e. pre-fish subpopulation), followed by a period with high fish
115 predation (i.e. high-fish subpopulation), and finally a period with relaxed fish predation (i.e.
116 reduced-fish subpopulation) (Cousyn et al., 2001; Stoks, Govaert, Pauwels, Jansen, & De
117 Meester, 2016). We have previously shown that the changes of fish predation pressure were
118 associated with rapid, partly reversed evolution of phenotypic trait means and plasticity for a
119 set of life history, morphology and behavioural traits (Stoks et al., 2016).

120 In the current study, we characterized the metabolomic profiles of 18 clones (6 clones
121 from each of the three subpopulations differing in fish predation pressure) of *D. magna* both

122 in the absence and in the presence of fish kairomones. We first determined how predation risk
123 changes the metabolomic profile of *D. magna*, i.e. which metabolites and metabolic pathways
124 were affected by predation risk. We then partitioned the total changes of metabolomic
125 profiles between successive periods in fish predation to quantify the relative contributions of
126 ancestral plasticity, constitutive evolution, and evolution of plasticity (Stoks et al., 2016).
127 Utilizing these data, we further tested whether and how plastic responses and evolutionary
128 responses covaried with each other, testing the ideas whether metabolites that show the
129 strongest ancestral plastic responses to fish kairomones also show the strongest evolutionary
130 responses, or, alternatively, that these responses are negatively correlated (Huey, Hertz, &
131 Sinervo, 2003). These analyses enabled us to get unique insights in the plasticity and
132 evolution of the metabolome in a natural population in response to a novel stressor.

133 **Materials and Methods**

134 **Resurrection of *Daphnia* clones and culture conditions**

135 *Daphnia magna* clones were hatched from dormant eggs of three sediment layers of a
136 shallow lake in Belgium (50°50' N, 4°39' E, Oud-Heverlee, Belgium). The three sediment
137 layers match specific periods differing in fish predation pressure (Cousyn et al., 2001): the
138 pre-fish period (1970 - 1972) with no fish present, the high-fish period (1976 - 1979) with
139 high fish predation pressure because of intensive fish stocking (> 300 kg/ha), and the
140 reduced-fish period (1988 - 1990) with relaxed fish predation pressure because of reduced
141 fish stocking. An analysis of microsatellite variation indicated that the *Daphnia* from
142 different periods can be considered as belonging to different subpopulations of one
143 continuous population (Cousyn et al., 2001). For current study, six clones per period were
144 used. To minimize interference from maternal effects, all clones were cultured under standard
145 conditions for three generations prior to the experiment: up to 18 adults in 500 mL glass

146 vials, no fish kairomones, 20 °C, photoperiod 14:10 light:dark, daily fed *S. obliquus* (1.5 ×
147 10^5 cells mL^{-1}, ~1.25 mg C L^{-1}) and with renewal of the culture medium every other day.

148 **Experimental set-up**

149 We tested for effects of fish kairomones, subpopulation and their interaction on the
150 metabolomes in a full factorial experiment. The total design consisted of 6 clones × 3
151 subpopulations × 2 fish kairomone treatments × 8 replicates = 288 experimental units. To
152 manipulate fish predation risk, fresh medium containing fish kairomones was added daily. To
153 prepare the medium, three fish (5-7 cm *Gasterosteus aculeatus* sticklebacks) were kept for 24
154 h in 20 L aerated and bio-filtered tap water. This fish-conditioned water was filtered twice
155 (0.45 µm) and diluted five times to obtain a final concentration of three fish per 100 L, which
156 is known to generate strong responses in *D. magna* (Pauwels, Stoks, & De Meester, 2010;
157 Zhang, Jansen, De Meester, & Stoks, 2016). The fish were fed *D. magna* daily in a separate
158 bucket to avoid the presence of *Daphnia* alarm cues in the fish medium. The culture medium
159 was refreshed every other day.

160 To obtain enough synchronized juveniles to start the experiment, for each clone we
161 cultured ten to twelve *Daphnia* mothers from one grandmother. Cohorts of 16-18 juveniles of
162 the pooled second brood of these mothers, all born within a 24 h interval, were used as
163 experimental animals and cultured in 500 mL glass vials filled with 450 mL bio-filtered tap
164 water. For each clone there were 8 replicates, hence 8 vials with 16-18 juveniles. To obtain
165 relatively synchronised individuals for metabolomic profiling, one set of three individuals per
166 vial were collected that had released their second clutch and had no visual signs of the third
167 clutch in the brood pouch. Because the first clutch typically is small and not responsive to
168 fish cues compared to the second clutch (Stoks et al., 2016), the physiological status and
169 metabolome of the mother *Daphnia* might be more influenced by fish predation after release

170 the second clutch. When sampling, *Daphnia* were quickly rinsed with deionized water before
171 transferring into a 1.5 ml centrifuge tube; the remaining water was gently removed with a
172 glass Pasteur pipet. All samples were flash frozen in liquid nitrogen and then stored at -80 °C.

173 **Metabolomic profiling**

174 The metabolome of *Daphnia* samples was analysed at the Natural Environment Research
175 Council (NERC) Biomolecular Analysis Facility at the University of Birmingham (UK). The
176 metabolome of *Daphnia* samples were analysed using nano-electrospray ionization - direct
177 infusion mass spectrometry (nESI-DIMS) as described by Southam *et al.* (2017) in their
178 Supporting Information. Here, we applied nano-electrospray direct infusion mass
179 spectrometry as this approach has been demonstrated to provide the required sensitivity when
180 working with low biomass *Daphnia* samples and thereby allows running 3-4 technical
181 replicates per sample (Southam et al., 2017; Taylor, Gavin, & Viant, 2018). Briefly, polar
182 metabolites were first extracted from *D. magna* samples using a biphasic method. Then, all
183 samples were analysed in both positive and negative ionisation modes using an Orbitrap Elite
184 mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) with a direct infusion, chip-
185 based nano-electrospray ionization source (Triversa, Advion Biosciences, Ithaca, NY, USA).
186 The data processing was done using the Galaxy online platform using the selected ion
187 monitoring (SIM) stitching algorithm and data were acquired from mass to charge ratios
188 (m/z) in the range 50–620 (Southam et al., 2017). After several steps (replicate filter, blank
189 filter, sample filter, see details in Supporting Information), the processed data matrices were
190 used for bioinformatics and statistical analyses.

191 **Statistical analyses of metabolomic profiles**

192 To assess the effects of exposure to fish kairomones and subpopulation on the metabolome of
193 *D. magna*, we applied two parallel multivariate analyses: a principal component analysis

194 (PCA) coupled with two-way ANOVA and an ANOVA-simultaneous component analysis
195 (ASCA). The results of both analyses were very similar and here we only show the PCA-
196 ANOVA results (the ASCA results are in the Supplementary Information). PCA was
197 conducted on the processed data matrices to assess the broad-scale variation between the two
198 treatments using the PLS Toolbox (version 5.5.1, Eigenvector Research, Manson, WA, USA)
199 within Matlab (version 7.8; The MathsWorks, Natick, MA, USA) following mean centring of
200 the processed DIMS data. We extracted the first two PC axes for both ion modes; these
201 explained 43.1 % (positive ion mode) and 42.9 % (negative ion mode) of the total variation.
202 In order to test the effects of fish kairomones and subpopulation on the metabolome of *D.*
203 *magna*, we then applied two-way ANOVAs on the generated PC scores in Statistica v12.0. In
204 each analysis, the fish kairomone treatment, subpopulation and their interaction were
205 included as fixed factors and clone was nested in subpopulation as a random factor. A
206 significant effect of the fish kairomone treatment indicates plasticity, while a subpopulation
207 effect indicates rapid evolution of the trait means, and a fish kairomone × subpopulation
208 interaction indicates rapid evolution of plasticity.
209 As we found strong fish kairomone × subpopulation interactions on the metabolome,
210 we then applied partial least squares discriminant analysis (PLS-DA) to each subpopulation
211 separately to identify the specific metabolic responses to fish kairomones for each
212 subpopulation. PLS-DA uses prior knowledge of the sample classes (here the fish kairomone
213 treatments) to maximize separation of the metabolic profiles of the different classes and to
214 derive predictive models (Nicholson, Connelly, Lindon, & Holmes, 2002). Internal cross-
215 validation and permutation testing were employed to prevent over-fitting of the data
216 (Westerhuis et al., 2008). Putative marker metabolites in response to fish kairomones for each
217 subpopulation were screened using as criterion a Variable Importance in Projection (VIP)

218 threshold greater than 1 (Xuan et al., 2011). All putative marker metabolites for each
219 subpopulation were compared to screen for the general and subpopulation-specific metabolite
220 responses to fish kairomones. PLS-DA was conducted using in-house scripts with the PLS-
221 Toolbox in Matlab.

222 In addition, changes in the intensities of individual m/z peaks were also assessed
223 using t-tests for each subpopulation separately. All t-tests were corrected using a false
224 discovery rate (FDR, Benjamini & Hochberg, 1995) of 5% to account for multiple testing and
225 adjusted p-values are reported. Differences in the number of significantly changed peaks
226 among subpopulations were tested using a chi-square test. The effects of fish kairomones and
227 subpopulation on the metabolic rate was analysed using a two-way ANOVA in Statistica
228 v12.0 (Stat-soft, Tulsa, OK, USA).

229 **Metabolites annotation and pathway analyses**

230 We used the MI-Pack software (Weber & Viant 2010) and the KEGG (Kyoto Encyclopedia
231 of Genes and Genomes) platform to annotate the metabolites. The m/z peaks were assigned to
232 possible, putative empirical formula(e) and KEGG compound names based on MI-Pack
233 calculations. We then used MetaboAnalyst (Xia & Wishart, 2011) to analyse the metabolic
234 pathways that were affected by fish kairomones. We put all putatively annotated KEGG
235 compounds with VIP scores > 1 (based on the PLS-DA model including all three
236 subpopulations) into MetaboAnalyst for metabolic pathway visualisation. Fisher's exact tests
237 were used for over-representation analysis (Toyota, Gavin, Miyagawa, Viant, & Iguchi,
238 2016) and out-degree centrality was used for pathway topology analysis (Xia & Wishart,
239 2011). The FDR-corrected p values and impact values of all annotated pathways were
240 plotted. Pathways were filtered based on the uncorrected p values (-log p > 0.5) and impact
241 value (> 0.2) as those pathways were considered as potentially affected (Ratnasekhar,

242 Sonane, Satish, & Mudiam, 2015). We also used "MS peaks to pathways" in MetaboAnalyst
243 (Chong et al., 2018) to detect the affected metabolic pathways in each subpopulation
244 separately.

245 **Phenotypic trajectory analysis**

246 We applied phenotypic trajectory analysis (PTA) to test whether the magnitude and direction
247 of the multivariate plastic response of the metabolome to fish kairomones differed among
248 subpopulations. This technique tests for pairwise differences between groups in multivariate
249 plasticity (i.e. the phenotypic trajectories) by comparing the magnitude and the direction of
250 the two-state multivariate reaction norms (Collyer, Adams, & Biology, 2007). PTA allows
251 statistical testing for differences in magnitude and direction of phenotypic change by
252 comparing observed values to distributions created from random pairs of trajectories obtained
253 by permutations (Collyer et al., 2007). We compared the multivariate plasticity using all
254 important metabolite peaks (VIP > 1) identified by the PLS-DA model including all three
255 subpopulations.

256 The detailed methods of the PTA analyses are presented in the Supplementary
257 Information. Briefly, we tested for differences in the magnitude and direction of the
258 multivariate metabolomic change among the subpopulations using an extended R script of
259 Adams & Collyer (Adams & Collyer, 2009) where the statistical model included fish
260 kairomones, subpopulation and their interaction, and effects of clonal variation. To visualize
261 the multivariate reaction norms, we conducted a principal component analysis, and plotted
262 the scores on the first three, varimax normalized components. Note that these bivariate
263 projection PCA plots cannot fully reflect the magnitudes and angles of the multivariate
264 reaction norms as the PTA is conducted in a multi-dimensional trait space (Collyer et al.,
265 2007).

266 **The relative contribution of plasticity and evolution to metabolite changes**

267 For both transitions between two successive periods differing in fish predation pressure we
268 calculated the total peak change of important metabolites (VIP >1), i.e. the peak change as
269 would be observed when comparing the *Daphnia* under the period-specific kairomone
270 treatments (absence of fish kairomones in the pre-fish and reduced-fish periods, and presence
271 of fish kairomones in the high-fish period), following the method described in (Stoks et al.,
272 2016). We partitioned the total change for each important metabolite into three components:
273 ancestral plasticity, constitutive evolution, and evolution of plasticity (see Figure 1 for
274 details). Ancestral plasticity refers to the plasticity present in the older period of a given
275 transition. Constitutive evolution refers to evolution of the mean in the 'ancestral' kairomone
276 condition for a given transition between periods, hence in the absence of fish kairomones
277 when going from the no-fish to the high-fish period and in the presence of fish kairomones
278 when going from the high-fish to the reduced-fish period. Evolution of plasticity refers to the
279 change in the slope of the reaction norm and is the remainder of the total trait change after
280 subtracting ancestral plasticity and constitutive evolution from total phenotypic trait change
281 (see Figure 1). Pairwise t-tests were conducted to compare the contributions of evolution of
282 plasticity and of constitutive evolution to the total evolutionary change during both
283 transitions.

284 **The relationships between ancestral plasticity and evolutionary responses**

285 For both transitions, we explored the relationships between ancestral plasticity and
286 evolutionary changes (i.e., ancestral plasticity vs. total evolutionary changes, ancestral
287 plasticity vs. evolution of plasticity, and ancestral plasticity vs. constitutive evolution). We
288 therefore performed Pearson's correlations between these components for all the identified
289 important (VIP >1) metabolite peaks.

290 **Results**

291 **General metabolomic responses**

292 After mass spectral data processing, 2152 metabolites for the positive ion mode and 1519
293 metabolites for the negative ion mode were retained. The PCA results showed that fish
294 kairomones induced a clear separation of the metabolomes along PC2 (Figure 2a, b). While
295 there was also a separation along PC1, this was not linked to the treatments or subpopulations
296 (Figure S1). Further two-way ANOVA on the PC2 scores showed significant effects of the
297 fish kairomone and the fish kairomone × subpopulation interaction (all $P < 0.001$, Table S1).
298 These significant effects were confirmed by the ASCA model results (all $P < 0.0001$, Figure
299 S2). Because of the significant fish kairomone × subpopulation effects, PLS-DA analyses for
300 each subpopulation were conducted separately, and the results confirmed that the fish
301 kairomone treatment significantly changed the metabolomes in each subpopulation (all $P <$
302 0.001, Figure 2c-h, Table S2). Note that there was separation not only in LV1 but also in LV2
303 in the high-fish subpopulation (Figure 2e, f).

304 **Metabolites annotation and pathway analyses**

305 MI-Pack annotation showed 24.35 % (524 of 2152) of the peaks from the positive ion mode
306 and 37.72 % (573 of 1519) of those from the negative ion mode could be assigned at least
307 one molecular formula. Most of the annotated peaks were free amino acids and free sugars.
308 The lists of the putatively annotated peaks are shown in the supplementary datasets 1 and 2.
309 Analysing these putatively annotated peaks using MetaboAnalyst showed that predation risk
310 generally affected four metabolic pathways (valine, leucine valine and isoleucine valine
311 biosynthesis, D-glutamine and D-glutamate metabolism, purine metabolism, arginine and
312 proline metabolism) for the positive ion mode and five pathways for the negative ion mode
313 (starch and sucrose metabolism, biotin metabolism, galactose metabolism, ubiquinone and

314 other terpenoid-quinone biosynthesis, valine, leucine and isoleucine biosynthesis), which
315 were mostly linked to amino acid and sugar metabolism (Figure 3).
316 When analysing each subpopulation separately, we found the most enriched pathways
317 were different between subpopulations. For the positive ion mode, the pathway of 'valine,
318 leucine and isoleucine biosynthesis' was mostly enriched in the pre-fish subpopulation while
319 the pathway of 'arginine and proline metabolism' was mostly enriched for the high-fish and
320 reduced-fish subpopulations (Table S3). For the negative ion mode, the pathway of
321 'phenylalanine metabolism' was mostly enriched for the pre-fish subpopulation, the pathway
322 of 'cysteine and methionine metabolism' was mostly enriched for high-fish subpopulation,
323 and the pathway of 'alanine, aspartate and glutamate metabolism' was mostly enriched for
324 the reduced-fish subpopulation (Table S4).
325 **Subpopulation-specific metabolomic profiles under predation risk**
326 The metabolomic responses to fish kairomones differed strongly among subpopulations. Only
327 a small part of the responsive peaks was shared by all three subpopulations: 15.9 % (176 of
328 1105, Figure 4a) for the positive ion mode, and 23.5 % (136 of 580, Figure 4b) for the
329 negative ion mode. The total number of peaks whose levels were differentially modulated in
330 response to fish kairomones differed among the subpopulations (positive ion mode: x_2^2 =
331 278.39, $P < 0.001$; negative ion mode: x_2^2 = 79.40, $P < 0.001$, Table S5). The high-fish
332 subpopulation had the highest number of responsive peaks (Figure 4, Table S5), indicating
333 that the metabolome of the high-fish subpopulation changed most strongly in response to fish
334 kairomones.
335 Projecting the phenotypic trajectories onto the metabolomic PCA landscape showed that
336 for both ion modes the magnitude of the multivariate metabolomic reaction norm was greater
337 for the high-fish subpopulation than the pre-fish ($P < 0.001$ for both ion modes) and the

338 reduced-fish ($P = 0.026$ for the positive, $P < 0.001$ for the negative ion mode)
339 subpopulations, while the latter two did not differ in magnitude (Figure 5a-d, Table S6). The
340 direction of the multivariate plasticity differed considerably between the pre-fish
341 subpopulation and the two other subpopulations (both $P < 0.001$). In contrast, the high-fish
342 and reduced-fish subpopulations did not differ in the direction of multivariate plasticity for
343 the positive ion mode ($P = 0.086$), and only differed slightly for the negative ion mode ($P =$
344 0.047) (Table S6).

345 **Contributions of plasticity and evolution to total metabolomic changes in time**

346 For the positive ion mode, ancestral plasticity had about an equal contribution to the total
347 metabolomic change compared to the evolutionary components during both transitions in fish
348 predation (47.3% in the first transition and 55.9% in the second transition, Figure 6a-b). Of
349 the two evolutionary components, the contribution of evolution of plasticity was larger
350 compared to constitutive evolution during both transitions (pre-fish to high-fish: 31.0% vs
351 21.7%, t= -7.88, df = 517, $P < 0.0001$; high-fish to reduced-fish: 24.6% vs 19.4%, t = -5.41,
352 df = 517, $P < 0.0001$). The results were highly similar for the negative ion mode: ancestral
353 plasticity had about an equal contribution compared with evolution during both transitions
354 (46.5% and 48.8%). Of the two evolutionary components, the evolution of plasticity
355 contributed more than constitutive evolution during pre-fish to high-fish transition (30.4% vs
356 23.1%, t = -6.14, df = 405, $P < 0.0001$) and high-fish to reduced-fish transition (30.0% vs
357 21.2%, t = -7.46, df= 405, $P < 0.0001$) (Figure 6c-d).

358 **Relationships between ancestral plasticity and the evolutionary responses**

359 For the positive ion mode, the ancestral plasticity in the pre-fish subpopulation correlated
360 positively with the subsequent total evolution during the transition from pre-fish to high-fish
361 (R = 0.28, $P < 0.0001$, Figure 7a). This pattern was driven by the evolution of plasticity (R =

362 0.43, $P < 0.0001$, Figure 7c), while the correlation of ancestral plasticity with constitutive
363 evolution was negative (R = -0.28, $P < 0.0001$, Figure 7e). During the transition from high-
364 fish to reduced-fish these patterns were reversed: the correlations with ancestral plasticity
365 were negative for both total evolution (R = -0.43, $P < 0.0001$, Figure 7b) and evolution of
366 plasticity (R= -0.55, $P < 0.0001$, Figure 7d), and positive with constitutive evolution (R =
367 0.39, $P < 0.0001$, Figure 7f). These patterns were largely similar for the negative ion mode
368 (Figure S5).

369 **Discussion**

370 Using resurrection ecology, we provide unique evidence that changes in predation pressure
371 can drive rapid evolution of metabolomes and their plasticity in a natural prey population.
372 The high-fish subpopulation of *D. magna* evolved the strongest metabolomic response to
373 predation risk thereby matching the changes in fish predation pressure across periods and the
374 previously documented adaptive changes in life history, morphology and behaviour (Stoks et
375 al., 2016). Key findings about the interplay of plasticity and evolution were (i) that ancestral
376 plasticity and evolution contributed nearly equally in driving total metabolomic changes
377 through time with the evolution of plasticity being the larger evolutionary component, (ii) and
378 that the ancestral plasticity in the metabolome covaried positively with evolution of plasticity
379 when predation pressure increased while this pattern reversed with subsequent relaxation of
380 predation pressure.

381 **Predator-induced plastic changes in metabolomic profiles**

382 The mechanisms underlying the widespread trait responses to predation risk are still poorly
383 understood (Mitchell, Bairos-Novak, & Ferrari, 2017). Using metabolomics, we found that
384 the mostly affected metabolites of *D. magna* under predation risk were free amino acids and
385 free sugars, and accordingly, amino acid metabolism (e.g., arginine and proline metabolism)

386 and sugar metabolism (e.g., starch and sucrose metabolism) pathways. These metabolic
387 changes under predation risk may reflect either more breakdown or more synthesis of the
388 associated polymers (proteins and carbohydrates). In the breakdown scenario, animals may
389 face energy limitation under predation risk leading to an increased breakdown of the
390 polymers to meet an increased energy demand. A recent meta-analysis of Rinehart &
391 Hawlena (2020) suggested that predation risk indeed tended to decrease the carbohydrate and
392 protein contents of prey to meet increased energy demands required to fuel stress responses.
393 The here studied *D. magna* population reduced the RNA:DNA ratio when exposure to fish
394 cues (Zhang et al., 2016), suggesting that the free amino acids changes may reflect an
395 increased protein catabolism. Alternatively, in the synthesis scenario, *D. magna* may not be
396 energy limited but re-allocate energy to other functions under predation risk. In support of
397 this, *Daphnia* maintains feeding and assimilation rates (Beckerman et al., 2007; Stibor &
398 Machacek, 1998) and even increases somatic growth rate (Stoks et al., 2016) under predation
399 risk, and the experiment was performed under saturating food concentrations. The changed
400 levels of free amino acids and sugars may therefore indicate more synthesis of proteins and
401 carbohydrates. For example, protein synthesis (e.g. Hsp) is known to increase under predation
402 risk to protect cells from protein malfunctioning in *D. magna* (Pauwels, Stoks, & De Meester,
403 2005; Pijanowska & Kloc, 2004). Further, exposure to fish kairomones has been shown to
404 upregulate genes involved in protein folding (an important step for protein synthesis) in *D.*
405 *magna* (Schwarzenberger, Courts, & von Elert, 2009).

406 **Predation drove adaptive metabolomic evolution**

407 By measuring the metabolomes of three subpopulations separated in time that belong to one
408 continuous population and underwent strong changes in fish predation pressure, we could
409 directly demonstrate rapid metabolomic evolution within a single natural population. The

410 three *D. magna* subpopulations did not seem to be equal in their 'metabolic fingerprint' not
411 only in the presence (Figure S3) but also in the absence (Figure S4) of fish kairomones,
412 suggesting rapid evolution of constitutive metabolic differences in this population. Besides
413 constitutive evolution, we also observed rapid evolution of metabolomic plasticity to
414 predation risk. Until now, it is poorly understood that whether and how the metabolome
415 evolves in natural populations. As a notable exception, the marine snail *Littorina littorea*
416 evolved different metabolomic responses to ocean acidification at much longer timescales
417 associated with postglacial range expansion, which was linked to regional adaptation in
418 physiology and life history (Calosi et al., 2017).

419 Two lines of evidence suggest that the rapid evolution of metabolomic responses to
420 predation risk was adaptive. First, in line with the highest fish predation pressure being
421 present in the high-fish subpopulation, this subpopulation showed a stronger metabolomic
422 response to fish kairomones compared to the pre-fish and reduced-fish subpopulations. This
423 was illustrated both by the high-fish subpopulation having the most metabolites responsive to
424 fish kairomones (Figure 4) and the largest magnitude of the multivariate metabolomic
425 reaction norm in response to fish kairomones (Figure 5). Notably, the fact that the high-fish
426 subpopulation had the highest number of unique metabolites whose levels were significantly
427 changed, might also have contributed to the separation in LV2 only being observed in the
428 high-fish subpopulation (Figure 2e, f). That the predator-induced changes in the levels of
429 these metabolites (particularly free amino acids and free sugars) are largest in the high-fish
430 period, therefore suggests these *Daphnia* evolved to mobilize more energy to defend
431 themselves against fish predation and associated predator-induced stress, which is consistent
432 with the adaptive evolution of higher predator-induced plastic changes in their phenotype
433 (life history, morphology and behaviour) (Stoks et al., 2016). Second, in line with fish

434 predators being only present in the high-fish and reduced-fish periods (Cousyn et al., 2001),
435 these two subpopulations had a more similar direction of the multivariate metabolomic
436 response under predation, while the pre-fish subpopulation differed to the high-fish and
437 reduced-fish subpopulations. Notably, also the mostly affected metabolic pathway under
438 simulated fish predation was the same in high-fish and reduced-fish subpopulations (arginine
439 and proline metabolism), and differed from the one in the pre-fish subpopulation (valine,
440 leucine and isoleucine biosynthesis, in the positive ion mode). As the free amino acids
441 arginine and proline can be used by mitochondria as a metabolic fuel under stress (Raza et al.,
442 2020), this may suggest that the high-fish and reduced-fish subpopulations evolved to better
443 utilize energy when coping with predation risk. This pattern is also consistent with the
444 multivariate reaction norms for life history, behaviour and morphology in a previous study
445 (Stoks et al., 2016).

446 Noteworthy, the metabolomic responses to fish kairomones in the reduced-fish
447 subpopulation did not fully convert back to those of the pre-fish subpopulation, similar to
448 what was observed for life history traits (Stoks et al., 2016). This illustrates also at the
449 metabolomic level that evolution in response to a new selective factor is not necessarily fully
450 reversed when that selection factor is relaxed (Hairston et al., 1999; Lahti et al., 2009).

451 **The interplay of plasticity and evolution in driving rapid metabolomic shifts through**
452 **time**

453 Partitioning the changes in metabolomic profiles during the two transitions in fish predation
454 pressure showed that ancestral plasticity contributed approximately equal to the total changes
455 in metabolite concentrations as evolution, i.e. the combination of constitutive evolution and
456 evolution of plasticity (Figure 6). This is consistent with the important contribution of
457 ancestral plasticity to the changes in life history and morphology in the studied system (Stoks

458 et al., 2016) and in other systems (Ghalambor et al., 2007). During both transitions, the
459 evolution of plasticity was more important than constitutive evolution. This is in line with the
460 expectation of evolutionary increases in plasticity in response to rapid increases of a novel
461 selection pressure (Chevin & Lande, 2015; Lande, 2009; Robinson, 2013). It, however,
462 deviates from earlier observations on phenotypic traits in this study system, for which
463 evolution of plasticity played a more important role when fish predation increased whereas
464 responses were more driven by constitutive evolution when predation was relaxed (Stoks et
465 al., 2016). Thus, the current study indicated that the evolution of the metabolome and the
466 evolution of phenotypic traits may partly be uncoupled. One possible reason for the
467 discrepancy between the evolutionary patterns in the metabolome and the previously reported
468 patterns in a set of 14 phenotypic traits, may be that the metabolome is thought to be linked to
469 the phenome (the total sum of phonotypic traits) (Sardans, Peñuelas, & Rivas-Ubach, 2011),
470 and we cannot exclude that other untested phenotypic traits actually follow the same pattern
471 as the metabolome.

472 During the first transition, when the prey population experienced a strong increase in
473 a novel selection agent (i.e. fish predation pressure), the ancestral plasticity and the evolution
474 of plasticity were positively correlated. In other words, metabolites that showed a stronger
475 plasticity to predation risk in the pre-fish subpopulation evolved to be even more plastic
476 under increased fish predation pressure. This pattern corroborates the interpretation that the
477 plastic response is adaptive and that evolution upon exposure to predation magnified the
478 plastic responses of metabolomes to this stressor in this natural population. While the positive
479 correlation between ancestral plasticity and evolution is consistent with the idea that ancestral
480 plasticity may facilitate adaptive evolution (Fox et al., 2019; Ghalambor et al., 2007; Levis &
481 Pfennig, 2016), an alternative explanation might be that plasticity in response to a stressor is

482 enhanced because it was adaptive and the stressor increased in strength. This latter
483 interpretation is more in line with the pattern that we observe in the second transition.
484 Notably, when the selection was relaxed again during the second transition, the sign
485 of the association reversed: the ancestral plasticity and evolution of plasticity were negatively
486 correlated, indicating that metabolites with a stronger plasticity to predation risk in the high-
487 fish subpopulation evolved to be less plastic under relaxed pressure. This suggests that the
488 stronger metabolomic plasticity under predation risk as observed in the high fish period is
489 costly and counter-selected against when predation pressure turns low. This matches the
490 reduced plasticity for multiple life-history, morphology and behaviour traits in *D. magna*
491 during the second transition (Stoks et al., 2016), as also has been observed in Trinidadian
492 guppies when prey shift to a situation with relaxed predation pressure (Westrick, Broder,
493 Reznick, Ghalambor, & Angeloni, 2019). Our results during the second transition match with
494 the only other study reporting a correlation between ancestral plasticity and evolution at the
495 molecular level. Trinidadian guppies transplanted from a site with high to low predation
496 pressure also showed a negative association between ancestral plasticity (in this case of gene
497 expression) and total evolution (Ghalambor et al., 2015). Ghalambor et al. argued the
498 ancestral plasticity in the high-predation site to be maladaptive in the guppy system, and the
499 most plastic transcripts to evolve reduced plasticity as a result of strong selection against non-
500 adaptive plasticity (Ghalambor et al., 2015). Instead, in our study the positive association
501 between ancestral plasticity and evolution of plasticity suggests adaptive plasticity in the
502 high-predation period. Hence, our results indicate that in the *Daphnia* population the negative
503 correlation between ancestral plasticity and evolution of plasticity upon relaxation of the
504 predation pressure is in line with a true reversal of the response, with those metabolites that

evolved the strongest phenotypic plasticity during the first transition now showing the strongest reduction in plasticity.

The evolutionary responses in metabolite expression in our study population were driven by the evolution of plasticity. Constitutive evolution was a relatively minor component of the evolutionary response, and showed opposite covariation patterns with ancestral plasticity. Specifically, during the first transition metabolites that responded more plastically in the ancestral population showed less evolution in their mean levels in the absence of predation risk. This is suggestive of a pattern where ancestral plasticity can buffer evolution (Price et al., 2003), but in practice should be integrated with the evolution of plasticity. During the first transition, it thus seems that metabolites with higher ancestral plasticity show stronger evolution of plasticity but combine this with a change in main trait value in the ancestral environment that is opposite to the (change in) plasticity. Also, for constitutive evolution, the response pattern for the second transition reflects a reversal. Overall, our results show that the association between ancestral plasticity and evolution is complex, can depend on which component of evolution one studies, and might critically depend on whether one considers an increase in selection pressure or a release from this selection pressure. Our study also advocates for an integrated approach in which one goes beyond interpreting patterns from a one-directional study or across taxa and populations.

Conclusions

Our resurrection ecology study of evolution in a natural population provides unique input at the metabolomic level to the ongoing debate on the relationships between ancestral plasticity and subsequent evolutionary changes (Levis & Pfennig, 2016). We addressed two important outstanding questions (Fox et al., 2019; Ghalambor et al., 2015, 2007; Levis & Pfennig, 2016; López-Maury et al., 2008). First, we showed ancestral plasticity and evolution to

contribute nearly equally in driving total metabolomic changes through time. Second, we demonstrated that evolution of plasticity magnified the ancestral plasticity when a new selection pressure was imposed. Such insights are important to advance our ability to understand and predict how populations deal with the new and strong selection pressures which they are increasingly dealing with.

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Data accessibility

Metabolomics data have been deposited at dryad (doi.org/10.5061/dryad.vdncjsxtm).

Author contributions

701 CZ, LDM, MV and RS developed the concept of the study; CZ performed the experiment
702 with the input from MJ and MV for the metabolomics part; CZ analysed the data with the
703 input from MJ and LG. CZ wrote the first version of the manuscript; LDM and RS
704 contributed substantially to revisions and all authors agreed on the final manuscript.

Tables and Figures

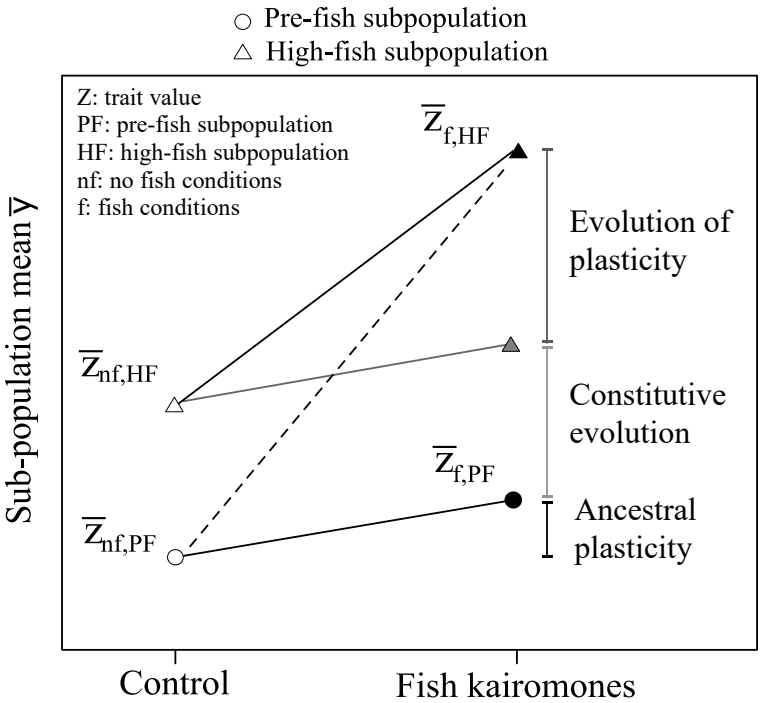

Figure 1 Methodology used to divide the total phenotypic trait change during a transition in fish predation pressure into three components: ancestral plasticity (= $\overline{Z}_{f,\,PF} - \overline{Z}_{nf,PF}$), constitutive evolution (= $\overline{Z}_{nf,\,HF} - \overline{Z}_{nf,PF}$) and evolution of plasticity (= $\overline{Z}_{f,\,HF} - \overline{Z}_{f,\,PF} - \overline{Z}_{nf,\,HF} + \overline{Z}_{nf,PF}$, see details in Stoks et al. 2016). Shown is the situation for the trait change between the pre-fish (●) and high-fish (▲) periods. White (black) symbols indicate subpopulation means in the absence (presence) of fish kairomones; the grey triangle represents the hypothetical trait value for the high-fish sub-population mean in the presence of fish kairomones in case only constitutive evolution and ancestral plasticity occur. The dashed line refers to the total trait change which is the trait change one would observe *in situ*.

Subpopulation	Control	Fish kairomones
Pre-fish	○	●
High-fish	△	▲
Reduced-fish	□	■

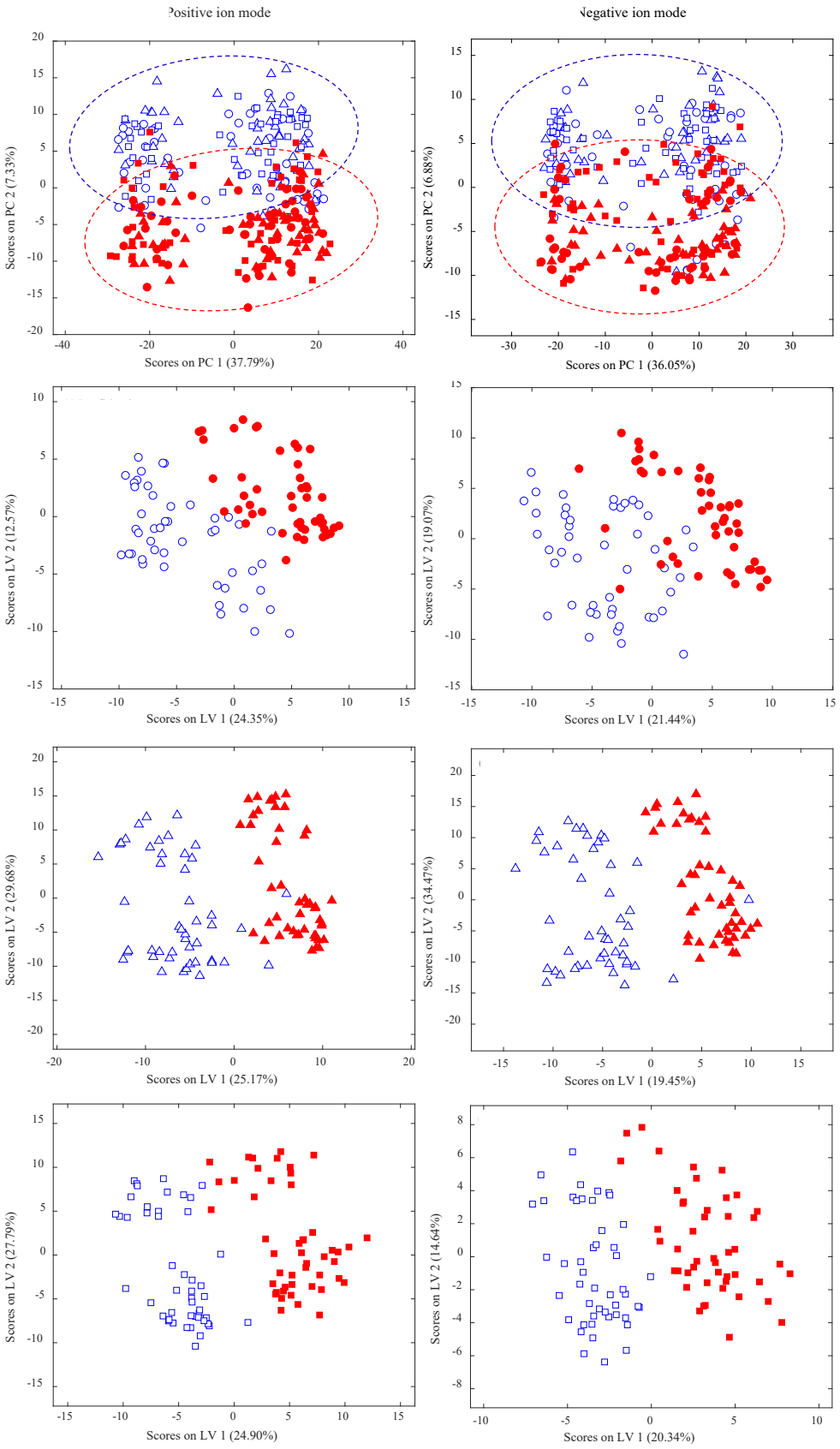

Figure 2 PCA and PLS-DA scores plots of positive (a, c, e, g) and negative (b, d, f, h) ion mode mass spectra describing the metabolomes of different subpopulations of *Daphnia magna* under control (blue colour) and fish predation risk (red colour). The ellipses of 95% confidence to the PCA plots were included in Figure 2a, b. LV1 and LV2 are the first two latent variables.

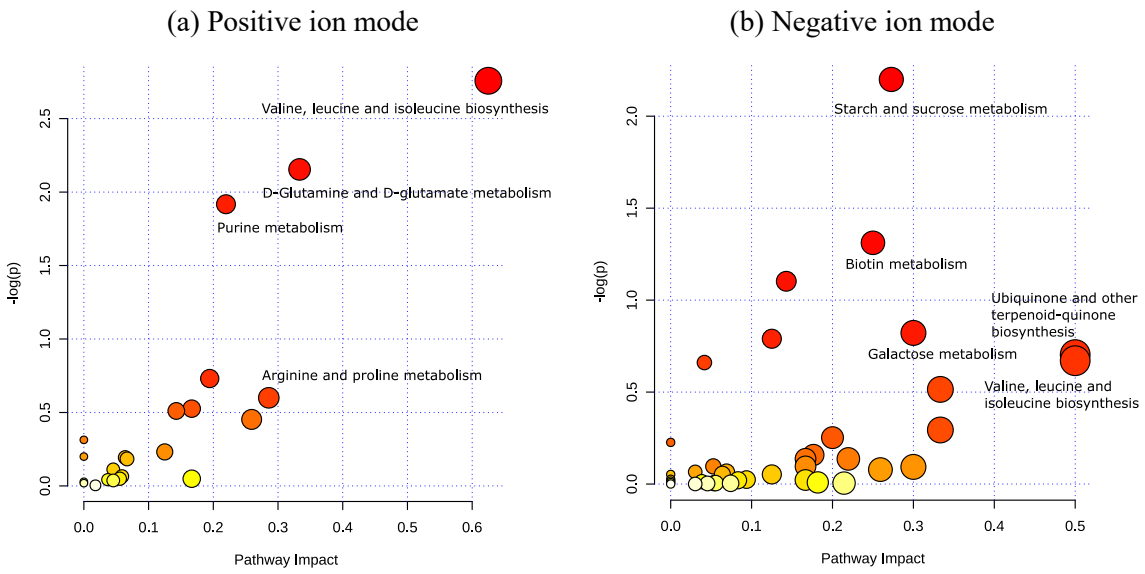

Figure 3 The identified metabolic pathways of *D. magna* that are most strongly reacting to fish kairomones. The circles represent pathways. The colour of each pathway is coded from white (lower impact value) to red (high impact value). The size of the circle is larger when pathway impact is higher. Only pathways with uncorrected p values (-log p > 0.5) and impact value (> 0.2) were labelled in the figure as those pathways were considered potentially affected.

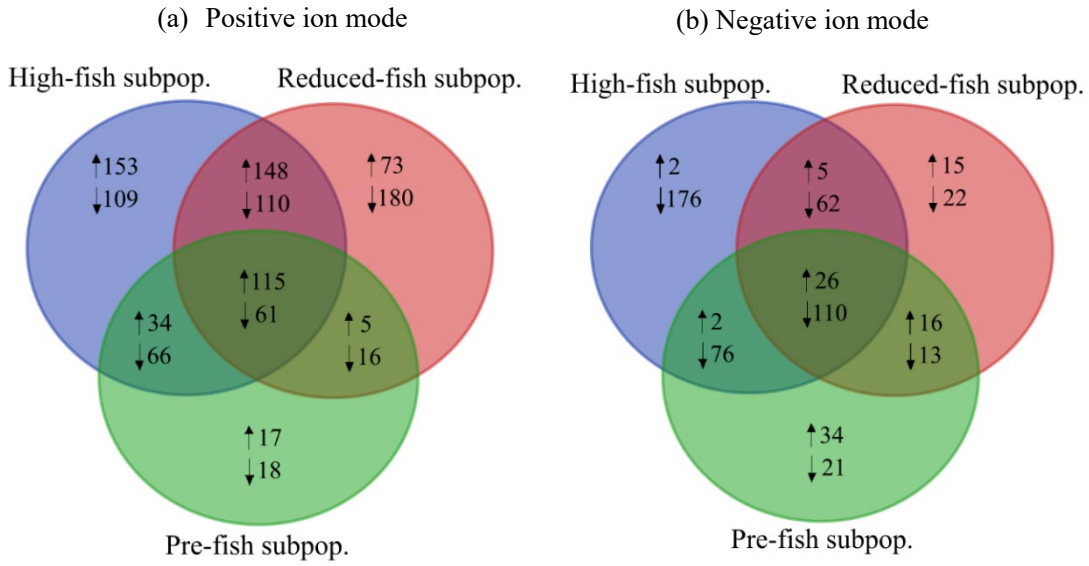

Figure 4 Venn diagram of the significantly up-modulated and down-modulated levels of metabolites in response to fish kairomones for each subpopulation of *Daphnia magna* for the (a) positive and (b) negative ion modes.

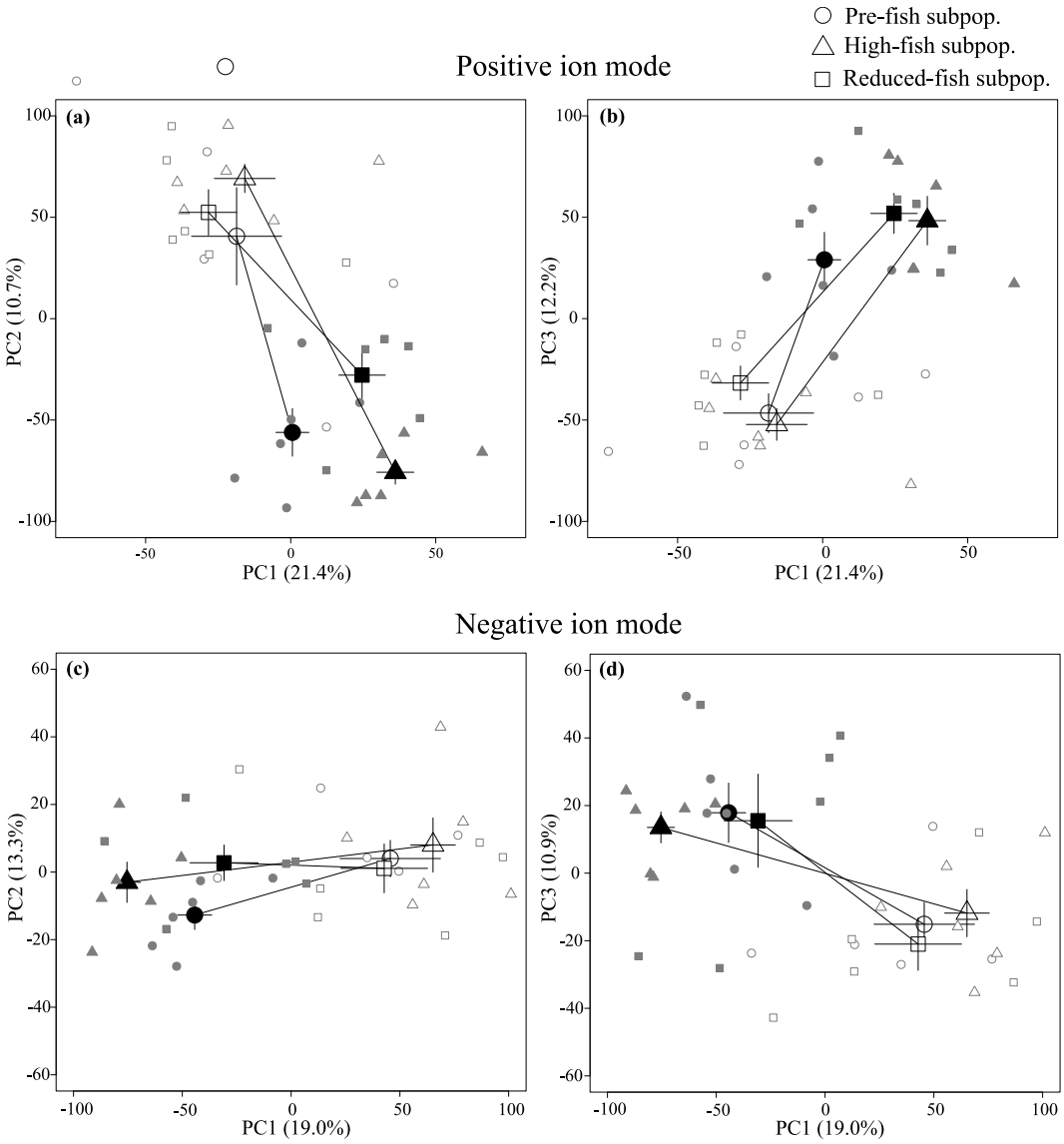

Figure 5 Multivariate metabolomic reaction norms representing the response to fish kairomones (open symbol: absence, filled symbol: presence) of the three subpopulations of *Daphnia magna*. Upper plots (a-b) show the patterns for the positive ion mode, lower plots (c-d) for the negative ion mode. Shown are patterns for PC1 and PC2 (a, c), and PC1 and PC3 (b, d).

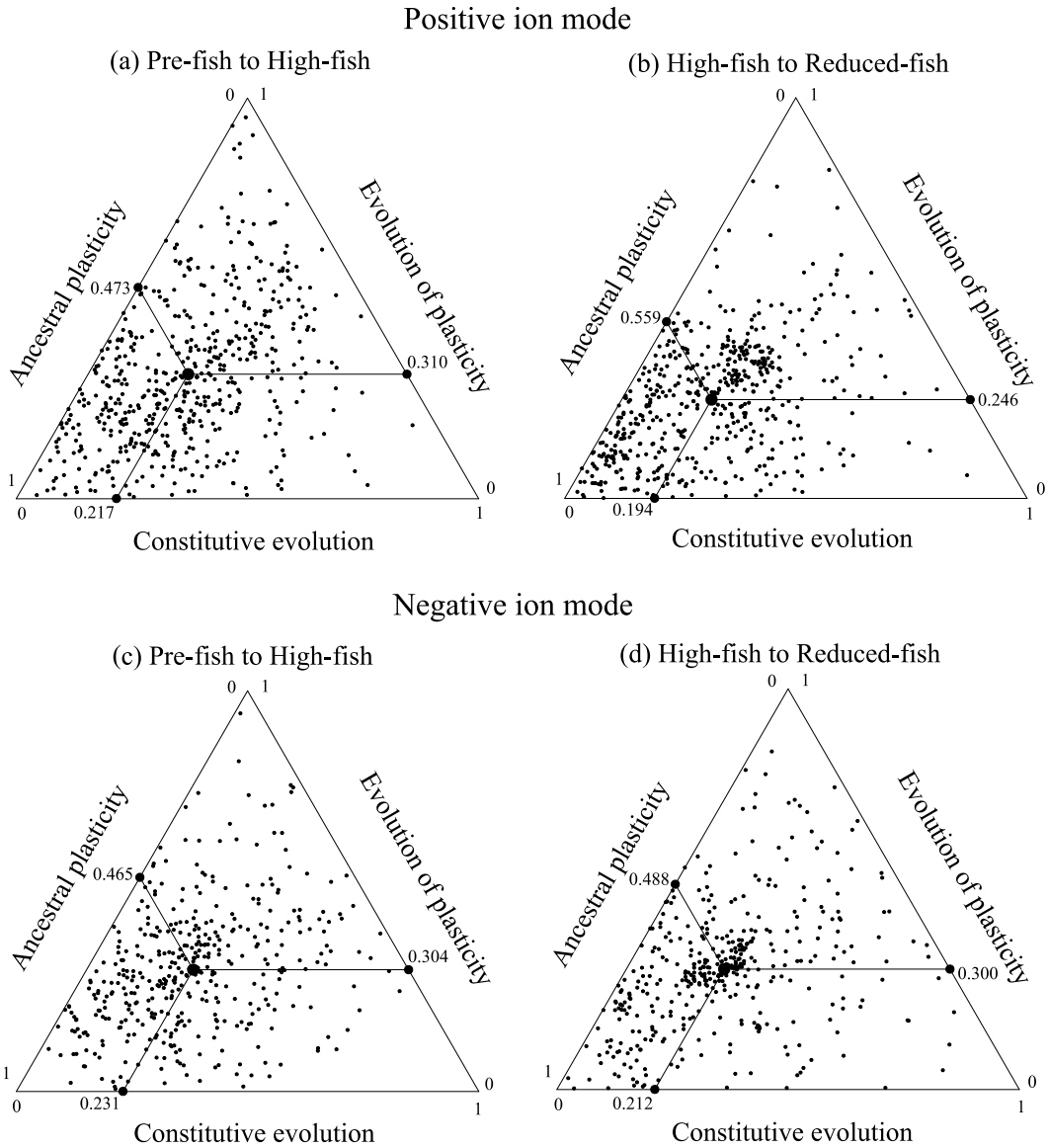

Figure 6 Relative contributions of ancestral plasticity, constitutive evolution and evolution of plasticity to the total changes in important metabolite peaks for (a, c) the transition from no fish to high fish predation and (b, d) the transition from high to reduced fish predation in the natural *D. magna* population. Partitioning was done separately for the positive ion mode (a, b; 518 peaks) and for the negative ion mode (c, d; 406 peaks). Shown are the results based on the partitioning method with contributions estimated using the additive method explained in Figure 1. Each small black dot represents a single metabolite peak. Large black dots represent the mean values of each component.

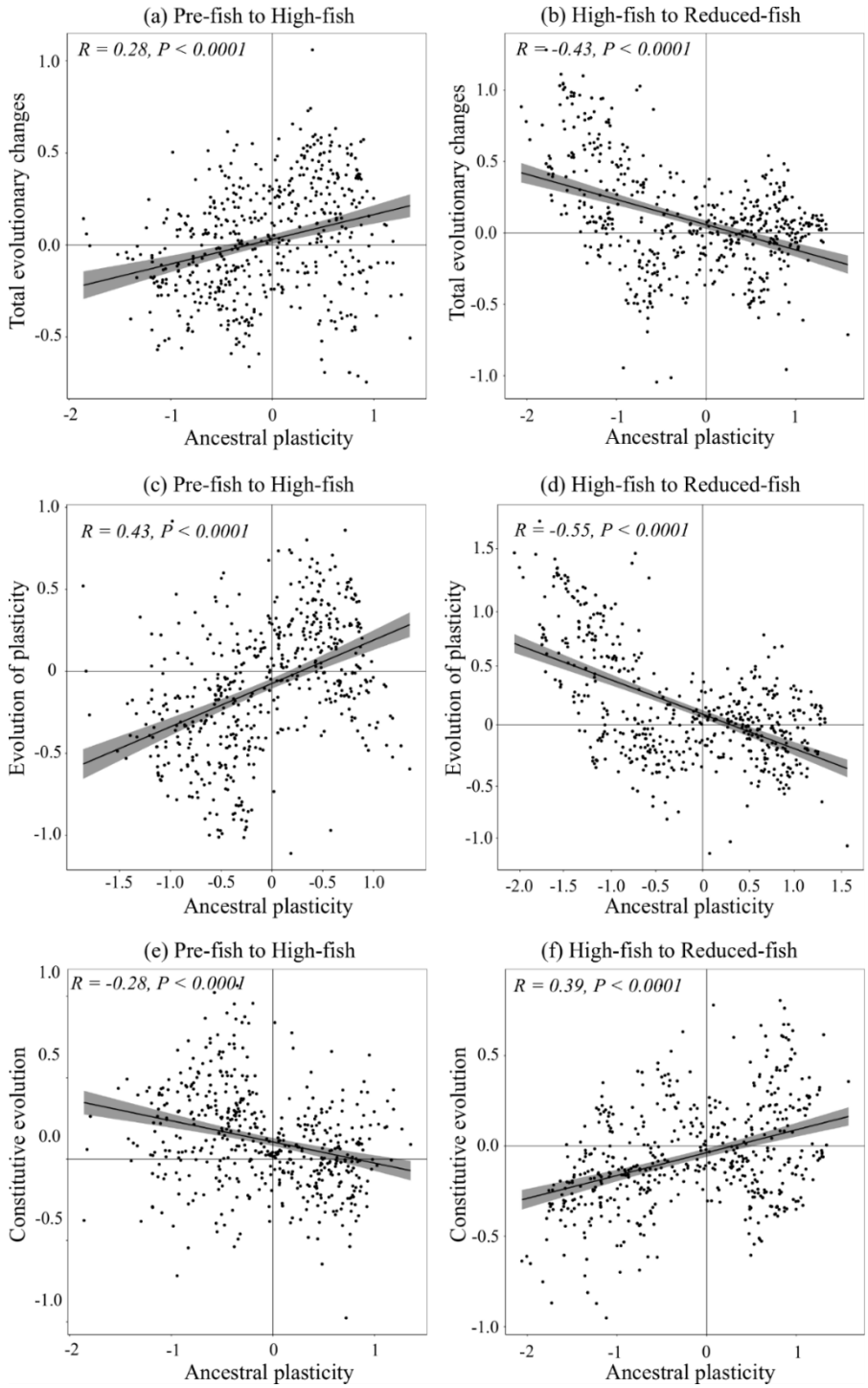

Figure 7 Relationships between ancestral plasticity and subsequent evolution of the metabolome: (a, b) total evolutionary changes; (c, d) evolution of plasticity; (e, f) constitutive evolution. Show are the patterns for both transitions in fish predation pressure for the positive ion mode (based on 518 peaks with VIP >1). Each dot represents a single metabolite peak. Pearson's correlations with *P*-values are given.