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

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Abstract

Populations rely on phenotypic plasticity and rapid evolutionary responses to adapt to novel environmental conditions. Because of the lack of compelling evidence from natural populations, controversy remains about the interplay between ancestral plasticity and rapid evolution in driving responses to new stressors. We studied this at the level of metabolome in a resurrected natural population of the water flea Daphnia magna that underwent an increase followed by a reduction in predation pressure within ˜16 years. Both the constitutive and plastic components of the metabolic profiles showed rapid adaptive evolution. Ancestral plasticity and evolution contributed nearly equally to the total changes of the metabolomes during both transitions. The metabolites with higher ancestral plasticity showed stronger evolution of plasticity when the predation pressure increased, while this pattern reversed when the predation pressure relaxed. Our results therefore highlight adaptive evolution in response to a new selection pressure in this natural population magnified the metabolomic plasticity to this stressor.

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 initial 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 (Ghalambor *et al.* 2007; Lande 2015; Fox*et al.* 2019). Against this background, important outstanding questions in current 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 (López-Maury *et al.* 2008; Ghalambor *et al.* 2015; Levis & Pfennig 2016; Fox *et al.* 2019). It is widely assumed that ancestral plasticity may facilitate adaptive evolution, hence ancestral plasticity and the subsequent evolutionary response may covary positively (Scoville & Pfrender 2010; Espinosa-Soto *et al.*2011). Yet, also the opposite has been hypothesized: that plasticity may slow down evolution by shielding traits from natural selection (Huey *et al.* 2003; Price *et al.* 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).

A key challenge when studying the interplay of plasticity and evolution in natural populations is to quantify the ancestral plasticity as often only the resulting phenotype after the joint action of plasticity and evolution

can be studied (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 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 *et al.* 2007; Weider *et al.* 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*et al.* 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 (Viant *et al.* 2017). Recently, metabolomics has been successfully applied to understand the plastic response to a variety of environmental stressors (Mayor *et al.* 2015; Taylor *et al.*2016; Calosi *et al.* 2017; Garreta-Lara *et al.* 2018). We, however, lack information on the rapid evolution and its interplay with plasticity of metabolomic profiles in natural populations.

One potent selective factor in natural populations is predation. There is widespread evidence that predators may generate not only plastic but also rapid evolutionary responses in a wide array of phenotypic traits in prey populations including life history (Reznick *et al.* 1997; Benard 2004), physiology (Hawlena & Schmitz 2010), morphology (Eklöv & Svanbäck 2017), and behaviour (Schoener *et al.* 2018). Yet, the metabolomic responses of prey (including both plastic and evolutionary responses) to predation remain unexplored.

Using a resurrection ecology approach, we aimed to test for rapid evolution of the metabolome and its plastic response to predation risk in a natural population of the water flea *Daphnia magna* that experienced strong and opposing transitions in fish predation pressure through a period of ˜16 years. First, there was a period with no fish predation, followed by a period with high fish predation, and finally a period with relaxed fish predation (Cousyn *et al.*2001; Stoks *et al.* 2016). This was associated with rapid, partly reversed evolution of phenotypic trait means and plasticity for a set of life history, morphology and behavioural traits (Stoks *et al.*2016).

Here, we characterized the metabolomic profiles of the clones from three subpopulations both in the absence and in the presence of predation risk. We found predation risk induced metabolomic plastic changes which were mainly related to shifts in amino acid and sugar metabolism, indicating predation risk affected protein utilization and energy supply. We then partitioned the total changes of metabolomic profiles between successive periods in fish predation to quantify the relative contributions of ancestral plasticity, constitutive evolution, and evolution of plasticity (Stoks *et al.* 2016). We found that ancestral plasticity and evolution (i.e. both constitutive evolution and evolution of plasticity) contributed nearly equally to the total changes of the metabolomes during both transitions. Using these data, we further explored whether and how plastic responses and evolutionary responses covaried with each other. The results showed that the metabolites with higher ancestral plasticity showed stronger evolution of plasticity when the predation pressure increased, while this pattern reversed when the predation pressure relaxed. This indicates that adaptive evolution in response to predation in this resurrected population magnified the metabolomic plasticity to this new stressor.

Materials and Methods

Resurrection of *Daphnia* clones and culture conditions

Daphnia magna clones were hatched from dormant eggs of three sediment layers of a shallow lake in Belgium (50°50' N, 4°39' E, Oud-Heverlee, Belgium). The three sediment layers match specific periods differing in fish predation pressure (Cousyn *et al.* 2001): the pre-fish period (1970 - 1972) with no fish present, the high-fish period (1976 - 1979) with high fish predation pressure because of intensive fish stocking (> 300 kg/ha), and the reduced-fish period (1988 - 1990) with relaxed fish predation pressure because of reduced fish stocking. An analysis of microsatellite variation indicated that the *Daphnia* from different periods can be considered as

belonging to different subpopulations of one continuous population (Cousyn*et al.* 2001). For current study, six clones per period were used. To minimize interference from maternal effects, all clones were cultured under standard conditions for three generations prior to the experiment: up to 18 adults in 500 mL glass vials, no fish kairomones, 20 °C, photoperiod 14:10 light:dark, daily fed *S. obliquus* (1.5×10^5 cells mL^{-1}, ~1.25 mg C L^{-1}) and with renewal of the culture medium every other day.

Experimental set-up

We tested for effects of fish kairomones, subpopulation and their interaction on the metabolomes in a full factorial experiment. The total design consisted of 6 clones × 3 subpopulations × 2 fish kairomone treatments × 8 replicates = 288 experimental units.

To manipulate fish predation risk, fresh medium containing fish kairomones was added daily. To prepare the medium, three fish (5-7 cm*Gasterosteus aculeatus* sticklebacks) were kept for 24 h in 20 L aerated and bio-filtered tap water. This fish-conditioned water was filtered twice (0.45 µm) and diluted five times to obtain a final concentration of three fish per 100 L, which is known to generate strong responses in *D. magna* (Pauwels *et al.* 2010). The fish were fed *D. magna* daily in a separate bucket to avoid the presence of*Daphnia* alarm cues in the fish medium. The culture medium was refreshed every other day. The medium refreshment was performed between 9:00 and 12:30 and feeding was done at 13:00 during the entire culturing period to maintain consistent.

To obtain enough synchronized juveniles to start the experiment, for each clone we cultured ten to twelve *Daphnia* mothers from one grandmother. Cohorts of 16-18 juveniles of the pooled second brood of these mothers, all born within a 24 h interval, were used as experimental animals and cultured in 500 mL glass vials filled with 450 mL bio-filtered tap water. During the experiment offspring was daily counted and removed from the vials. The experiment was ended when the animals had released their second clutch. The *Daphnia* developed relatively synchronously so that we could stop vials when all animals had released their second clutch and while there were no visual signs of the third clutch in the brood pouches. Animals were checked every 12 h and were not fed for the last 12 h before sampling to ensure empty guts. Per vial, we collected three individuals for metabolomic profiling. All samples were flash frozen in liquid nitrogen and then stored at -80 °C.

Metabolomic profiling

The metabolome of *Daphnia* samples were analysed at the Natural Environment Research Council (NERC) Biomolecular Analysis Facility at the University of Birmingham (UK). The metabolome of *Daphnia*samples were analysed using nano-electrospray ionization - direct infusion mass spectrometry (nESI-DIMS) as described by Southam *et al.* (2017). The detailed methods for the nESI-DIMS was in the supporting information. Briefly, polar metabolites were first extracted from *D. magna* samples using a biphasic method. Then, all samples were analysed in both positive and negative ionisation modes using an Orbitrap Elite mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) with a direct infusion, chip-based nano-electrospray ionization source (Triversa, Advion Biosciences, Ithaca, NY, USA). The data processing was done using the Galaxy online platform using the selected ion monitoring (SIM) stitching algorithm (Southam *et al.* 2017) (see supporting information for details). The processed data matrices were used for statistical analyses.

Statistical analyses

To assess the effects of fish kairomones and subpopulation on the metabolome of *D. magna* , we applied two parallel multivariate analyses: a principal component analysis (PCA) coupled with two-way ANOVA and an ANOVA-simultaneous component analysis (ASCA). The results of both analyses were very similar and here we only show the PCA - ANOVA results (see details for the ASCA results in the Supplementary Information). PCA was conducted on the processed data matrices to assess the broad-scale variation between the two treatments using the PLS Toolbox (version 5.5.1, Eigenvector Research, Manson, WA, USA) within Matlab (version 7.8; The MathsWorks, Natick, MA, USA) following mean centring of the processed DIMS data. We extracted the first two PC axes for both ion modes; these explained 43.1 % (positive ion mode)

and 42.9 % (negative ion mode) of the total variation. In order to test the effects of fish kairomones and subpopulation on the metabolome of*D. magna* , we then applied two-way ANOVAs on the generated PC scores in Statistica v12.0. In each analysis, the fish kairomone treatment, subpopulation and their interaction were included as fixed factors and clone was nested in subpopulation as a random factor. A significant effect of the fish kairomone treatment indicates plasticity, while a subpopulation effect indicates rapid evolution of the trait means, and a fish kairomone × subpopulation interaction indicates rapid evolution of plasticity.

As we found strong fish kairomone × subpopulation interactions on the metabolome, we applied partial least squares discriminant analysis (PLS-DA) to each subpopulation separately to identify the specific metabolic responses to fish kairomones for each subpopulation. PLS-DA uses prior knowledge of the sample classes (here the fish kairomone treatments) to maximize separation of the metabolic profiles of the different classes and to derive predictive models (Nicholson *et al.* 2002). Internal cross-validation and permutation testing (see details in Supplementary Information) were employed to prevent over-fitting of the data (Westerhuis *et al.* 2008). Putative marker metabolites in response to fish kairomones for each subpopulation were screened using as criterion a Variable Importance in Projection (VIP) threshold greater than 1 (Xuan *et al.* 2011). All putative marker metabolites for each subpopulation were compared to screen for the general and subpopulation-specific metabolites responsive to fish kairomones. PLS-DA was conducted using in-house scripts with the PLS-Toolbox in Matlab.

In addition, changes in the intensities of individual m/z peaks were also assessed using t-tests for each subpopulation separately. All t-tests were corrected using a false discovery rate (FDR, Benjamini & Hochberg, 1995) of 5% to account for multiple testing and adjusted p-values are reported. Differences in the number of significantly changed peaks among subpopulations were tested using a chi-square test.

Pathway analyses

We used MI-Pack and KEGG to annotate the metabolites (see details in the Supplementary Information). We then used MetaboAnalyst (Xia & Wishart 2011) to analyse the metabolic pathways that were affected by fish kairomones. We put all putatively annotated KEGG compounds with VIP scores > 1 (based on the PLS-DA model including all three subpopulations) into MetaboAnalyst for metabolic pathway visualisation. Fisher's exact tests were used for over-representation analysis (Toyota*et al.* 2016) and out-degree centrality was used for pathway topology analysis (Xia & Wishart 2011). The FDR-corrected p values and impact values of all annotated pathways were plotted. Pathways were filtered based on the uncorrected p values ($-\log p > 0.5$) and impact value (> 0.2) as those pathways were considered as potentially affected (Ratnasekhar *et al.* 2015).

Phenotypic trajectory analysis

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

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

The relative contribution of plasticity and evolution to metabolite changes

For both transitions between two successive periods differing in fish predation pressure we calculated the total peak change of important metabolites (VIP >1), i.e. the peak change as would be observed when comparing the *Daphnia* under the period-specific kairomone treatments (absence of fish kairomones in the pre-fish and reduced-fish periods, and presence of fish kairomones in the high-fish period), following the method described in (Stoks *et al.* 2016). We partitioned the total change for each important metabolite into three components: ancestral plasticity, constitutive evolution and evolution of plasticity (see Figure S1 in Supplementary Information). Ancestral plasticity refers to the plasticity present in the older period of a given transition. Constitutive evolution refers to evolution of the mean in the 'ancestral' kairomone condition for a given transition between periods, hence in the absence of fish kairomones when going from the no-fish to the high-fish period and in the presence of fish kairomones when going from the high-fish to the reduced-fish period. Evolution of plasticity refers to the change in the slope of the reaction norm and is the remainder of the total trait change after subtracting ancestral plasticity and constitutive evolution from total phenotypic trait change (see Figure S1).

The relationships between ancestral plasticity and evolutionary responses

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

Results

General metabolomic responses

We used mass spectrometry-based metabolomics to explore the metabolomes of *D. magna* . We obtained 2152 metabolites for the positive ion mode and 1519 metabolites for the negative ion mode. A PCA showed that fish kairomones induced a clear separation of the metabolomes along PC2 (Figure 1a, b). While there was also a separation along PC1, this was not linked to the treatments or clonal differences (see details in Supplementary Information). A two-way ANOVA on the PC2 scores showed significant effects of the fish kairomone treatment and the fish kairomone × subpopulation interaction (all $P < 0.001$, Table S1). These significant effects were confirmed by the ASCA model results (all $P < 0.0001$, see details in Supplementary Information). Because of the significant fish kairomone × subpopulation effect, we conducted separate PLS-DA for each subpopulation, and found the fish kairomone treatment significantly changed the metabolomes in each subpopulation (all$P < 0.001$, Figure 1c-h, Table S2).

Using MetaboAnalyst we found that four metabolic pathways for the positive ion mode and five pathways for the negative ion mode were affected by fish kairomones, these were mostly linked to amino acid and sugar metabolism (Figure 2).

Subpopulation-specific metabolomes under predation risk

The metabolomic responses to fish kairomones differed strongly among subpopulations. Only a small part of the responsive peaks was shared by all three subpopulations: 15.9 % (176 of 1105, Figure 3a) for the positive ion mode, and 23.5 % (136 of 580, Figure 3b) for the negative ion mode. The total number of differentially regulated peaks to fish kairomones differed among the subpopulations (positive ion mode: $x^2_2 = 278.39$, $P < 0.001$; negative ion mode: $x^2_2 = 79.40$, $P < 0.001$, Table S3). The high-fish subpopulation had the highest number of responsive peaks (Figure 3, Table S3), indicating that the metabolome of the high-fish subpopulation changed most strongly in response to fish kairomones.

Projecting the phenotypic trajectories onto the metabolomic PCA landscape showed that for both ion modes the magnitude of the multivariate metabolomic reaction norm was greater for the high-fish subpopulation than the pre-fish ($P < 0.001$ for both ion modes) and the reduced-fish ($P = 0.026$ for the positive, $P< 0.001$ for the negative ion mode) subpopulations, while the latter two did not differ in magnitude (Figure 4a-d, Table

S4). The direction of the multivariate plasticity differed considerably between the pre-fish subpopulation and the two other subpopulations (both$P < 0.001$). In contrast, the high-fish and reduced-fish subpopulations did not differ in the direction of multivariate plasticity for the positive ion mode ($P = 0.086$), and only differed slightly for the negative ion mode ($P = 0.047$).

Contributions of plasticity and evolution to total metabolomic changes in time

For the positive ion mode, ancestral plasticity had about an equal contribution to the total metabolomic change compared to the evolutionary components during both transitions in fish predation (47.3% in the first transition and 55.9% in the second transition, Figure 5a-b). Of the two evolutionary components, the contribution of evolution of plasticity was larger (31.0% and 24.6%) compared to constitutive evolution (21.7% and 19.4%) during both transitions. The results were highly similar for the negative ion mode: ancestral plasticity had about an equal contribution compared with evolution during both transitions (46.5% and 48.8%), and the evolution of plasticity contributed more (30.4% and 30.0%) than constitutive evolution (23.1% and 21.2%, Figure 5c-d).

Relationships between ancestral plasticity and the evolutionary responses

For the positive ion mode, the ancestral plasticity in the pre-fish subpopulation correlated positively with the subsequent total evolution during the transition from pre-fish to high-fish (R = 0.28, $P< 0.0001$, Figure 6a). This pattern was driven by the evolution of plasticity (R = 0.43, $P < 0.0001$, Figure 6c), while the correlation of ancestral plasticity with constitutive evolution was negative (R = -0.28, $P < 0.0001$, Figure 6e). During the transition from high-fish to reduced-fish these patterns reversed: the correlations with ancestral plasticity were negative for both total evolution (R = -0.43, $P < 0.0001$, Figure 6b) and evolution of plasticity (R= -0.55, $P < 0.0001$, Figure 6d), and positive with constitutive evolution (R = 0.39, $P< 0.0001$, Figure 6f). These patterns were largely similar for the negative ion mode (see details in Supplementary Information).

Discussion

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

Predator-induced plastic changes in metabolomic profiles

The mechanisms underlying the widespread trait responses to predation risk are still poorly understood (Mitchell *et al.* 2017). Our results clearly showed predation risk changed the metabolome of the prey*D. magna* . One key finding was that the amino acid metabolism (i.e. valine, leucine and isoleucine biosynthesis, and metabolism of beta-alanine, arginine and proline, tryptophan and phenylalanine) was strongly affected across subpopulations under predation risk. The pronounced changes in amino acid metabolism indicates *D. magna* deals with predation risk by altering protein utilization. This complements a proteomic study showing that exposure to predator kairomones enhanced the biosynthetic activity of proteins in *D. magna* , particularly glyceraldehyde-3-phosphate dehydrogenase (GAPDH) that is necessary for biosynthesis of amino acids (Otte *et al.* 2014). Related to this, exposure to fish kairomones has been shown to upregulate genes involved in protein folding in *D. magna* (Schwarzenberger *et al.*2009). In addition, the predator-induced change in amino acid metabolism may partly reflect changes in stress protein levels as documented for the study species (Pauwels *et al.* 2005). Besides playing a central role in the metabolic turnover of proteins, amino acids are also used extensively in energy metabolism (Viant *et al.* 2003). The proteomic study indeed showed evidence for enhanced energy demand of*D. magna* when under predation risk (Otte *et al.* 2014).

Predation drove adaptive metabolomic evolution

By measuring the metabolomes of three subpopulations separated in time that belong to one continuous population and underwent strong changes in fish predation pressure, we could directly demonstrate rapid metabolomic evolution within a single natural population. The three *D. magna*subpopulations differed in their 'metabolic fingerprint' not only in the presence (Figure S3) but also in the absence (Figure S4) of fish kairomones, suggesting rapid evolution of constitutive metabolic differences in this population. Besides constitutive evolution, we also observed rapid evolution of plastic metabolomic responses to predation risk. These patterns complement the rapid constitutive evolution and evolution of plastic responses to predation risk in life history, morphology and behaviour in this study system (Stoks *et al.*2016). We have poor knowledge on whether and how the metabolome evolves in natural populations. As a notable exception, the marine snail*Littorina littorea* evolved different metabolomic responses to ocean acidification at much longer timescales associated with postglacial range expansion, which was linked to regional adaptation in physiology and life history (Calosi *et al.* 2017).

Two lines of evidence suggest that the rapid evolution of metabolomic responses to predation risk was adaptive. First, the high-fish subpopulation showed a stronger metabolomic response to fish kairomones compared to the pre-fish and reduced-fish subpopulations. This was illustrated both by the high-fish subpopulation having the most metabolites responsive to fish kairomones and the largest magnitude of the multivariate metabolomic reaction norm in response to fish kairomones. Second, in line with fish predators being only present in the high-fish and reduced-fish periods (Cousyn *et al.* 2001), these two subpopulations had a more similar direction of the multivariate metabolomic response under predation compared to the pre-fish subpopulation. Also this pattern is consistent with the multivariate reaction norms for life history, behaviour and morphology (Stoks *et al.* 2016). Notably, the metabolomic responses to fish kairomones in the reduced-fish subpopulation did not fully convert back to those of the pre-fish subpopulation, similar to what was observed for life history traits in earlier work (Stoks *et al.* 2016). This illustrates also at the metabolomic level that evolution in response to a new selective factor is not necessarily fully reversed when that selection factor is relaxed (Lahti *et al.* 2009).

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

Partitioning the changes in metabolomic profiles during the two transitions in fish predation pressure showed that ancestral plasticity contributed approximately equal to the total changes in metabolite concentrations as evolution, i.e. the combination of constitutive evolution and evolution of plasticity. This is consistent with the important contribution of ancestral plasticity to the changes in life history and morphology in the studied system (Stoks *et al.* 2016) and in other systems (Ghalambor *et al.* 2007). During both transitions, the evolution of plasticity was more important than constitutive evolution. This in line with the expectation of evolutionary increases in plasticity in response to rapid increases of a novel selection pressure (Lande 2009; Robinson 2013; Chevin & Lande 2015). It, however, deviates from earlier observations on phenotypic traits in this study system, for which evolution of plasticity played a more important role when fish predation increased whereas responses were more driven by constitutive evolution when predation was relaxed (Stoks*et al.* 2016). This suggests that the evolution of the metabolome and the evolution of phenotypic traits may partly be uncoupled.

During the first transition, when the prey population experienced a strong increase in a novel selection agent (i.e. fish predation pressure), the ancestral plasticity and the evolution of plasticity were positively correlated. In other words, metabolites that showed a stronger plasticity to predation risk in the pre-fish subpopulation evolved to be even more plastic under increased fish predation pressure. This pattern corroborates the interpretation that the plastic response is adaptive and that evolution upon exposure to predation magnified the plastic responses of metabolomes to this stressor in this natural population. While the positive correlation between ancestral plasticity and evolution is consistent with the idea that ancestral plasticity may facilitate adaptive evolution (Ghalambor *et al.* 2007; Levis & Pfennig 2016; Fox *et al.* 2019), a more parsimonious explanation might be that plasticity in response to a stressor is enhanced because it was adaptive and the stressor increased in strength. This latter interpretation is more in line with the pattern that we observe in

the second transition.

Notably, when the selection was relaxed again during the second transition, the sign of the association reversed: the ancestral plasticity and evolution of plasticity were negatively correlated, indicating that metabolites with a stronger plasticity to predation risk in the high-fish subpopulation evolved to be less plastic under relaxed pressure. This suggests that the stronger metabolomic plastic response to predation risk as observed in the high fish period is costly and counter-selected against when predation pressure becomes low. This matches the reduced plasticity for multiple life-history, morphology and behaviour traits during the second transition (Stoks *et al.*2016), as also has been observed in other systems when prey shift to a situation with relaxed predation pressure (Westrick *et al.* 2019). Our results during the second transition seem to match with the only other study reporting a correlation between ancestral plasticity and evolution at the molecular level. Trinidadian guppies transplanted from a site with high to low predation pressure also showed a negative association between ancestral plasticity (in this case of gene expression) and total evolution (Ghalambor *et al.* 2015). Ghalambor et al. argued the ancestral plasticity in the high-predation site to be maladaptive in the guppy system, and the most plastic transcripts to evolve reduced plasticity as a result of strong selection against non-adaptive plasticity (Ghalambor *et al.* 2015). Instead, in our study the positive association between ancestral plasticity and evolution of plasticity clearly suggests adaptive plasticity in the high-predation period. Hence, our results suggest that in the*Daphnia* population the negative correlation between ancestral plasticity and evolution of plasticity upon relaxation of the 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 more plastic metabolites 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 (Ghalambor *et al.* 2007, 2015; López-Maury*et al.* 2008; Levis & Pfennig 2016; Fox *et al.* 2019). 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 might deal with the new and strong selection pressures they are increasingly dealing with.

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References

Adams, D.C. & Collyer, M.L. (2009). A general framework for the analysis of phenotypic trajectories in evolutionary studies.*Evolution* , 63, 1143–1154.

Benard, M.F. (2004). Predator-induced phenotypic plasticity in organisms with complex life histories. *Annu. Rev. Ecol. Evol. Syst.* , 35, 651–673.

Benjamini, Y. & Hochberg, Y. (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. R. Stat. Soc. B* ., 57, 289–300.

Calosi, P., Melatunan, S., Turner, L.M., Artioli, Y., Davidson, R.L., Byrne, J.J., *et al.* (2017). Regional adaptation defines sensitivity to future ocean acidification. *Nat. Commun.* , 8, 13994.

Chevin, L.M. & Lande, R. (2015). Evolution of environmental cues for phenotypic plasticity. *Evolution* , 69, 2767–2775.

Collyer, M.L., Adams, D.C. & Biology, O. (2007). Analysis of two-state multivariate phenotypic change in ecological studies. *Ecology* , 88, 683–692.

Cousyn, C., De Meester, L., Colbourne, J.K., Brendonck, L., Verschuren, D. & Volckaert, F. (2001). Rapid, local adaptation of zooplankton behavior to changes in predation pressure in the absence of neutral genetic changes. *Proc. Natl. Acad. Sci.* , 98, 6256–6260.

Eklöv, P. & Svanbäck, R. (2017). Predation risk influences adaptive morphological variation in fish populations. *Am. Nat.* , 167, 440.

Espinosa-Soto, C., Martin, O.C. & Wagner, A. (2011). Phenotypic plasticity can facilitate adaptive evolution in gene regulatory circuits. *BMC Evol. Biol.* , 11, 8–12.

Fox, R.J., Donelson, J.M., Schunter, C., Ravasi, T. & Gaitán-Espitia, J.D. (2019). Beyond buying time: The role of plasticity in phenotypic adaptation to rapid environmental change. *Philos. Trans. R. Soc. B Biol. Sci.* , 374.

Franks, S.J., Sim, S. & Weis, A.E. (2007). Rapid evolution of flowering time by an annual plant in response to a climate fluctuation.*Proc. Natl. Acad. Sci. U. S. A.* , 104, 1278–1282.

Garreta-Lara, E., Campos, B., Barata, C., Lacorte, S. & Tauler, R. (2018). Combined effects of salinity, temperature and hypoxia on*Daphnia magna* metabolism. *Sci. Total Environ.* , 610–611, 602–612.

Ghalambor, C.K., Hoke, K.L., Ruell, E.W., Fischer, E.K., Reznick, D.N. & Hughes, K.A. (2015). Non-adaptive plasticity potentiates rapid adaptive evolution of gene expression in nature. *Nature* , 525, 372–375.

Ghalambor, C.K., McKay, J.K., Carroll, S.P. & Reznick, D.N. (2007). Adaptive versus non-adaptive phenotypic plasticity and the potential for contemporary adaptation in new environments. *Funct. Ecol.* , 21, 394–407.

Hairston, N.G., Lampert, W., Cáceres, C.E., Holtmeier, C.L., Weider, L.J., Gaedke, U., *et al.* (1999). Rapid evolution revealed by dormant eggs. *Nature* , 401, 446.

Hawlena, D. & Schmitz, O.J. (2010). Herbivore physiological response to predation risk and implications for ecosystem nutrient dynamics.*Proc. Natl. Acad. Sci.* , 107, 15503–15507.

Huey, R.B., Hertz, P.E. & Sinervo, B. (2003). Behavioral drive versus behavioral inertia in evolution: A null model approach. *Am. Nat.* , 161, 357–366.

Lahti, D.C., Johnson, N.A., Ajie, B.C., Otto, S.P., Hendry, A.P., Blumstein, D.T., *et al.* (2009). Relaxed selection in the wild.*Trends Ecol. Evol.* , 24, 487–496.

Lande, R. (2009). Adaptation to an extraordinary environment by evolution of phenotypic plasticity and genetic assimilation. *J. Evol. Biol.* , 22, 1435–1446.

Lande, R. (2015). Evolution of phenotypic plasticity in colonizing species. *Mol. Ecol.* , 24, 2038–2045.

Levis, N.A. & Pfennig, D.W. (2016). Evaluating "plasticity-first" evolution in nature: Key criteria and empirical approaches. *Trends Ecol. Evol.* , 31, 563–574.

López-Maury, L., Marguerat, S. & Bähler, J. (2008). Tuning gene expression to changing environments: From rapid responses to evolutionary adaptation. *Nat. Rev. Genet.* , 9, 583–593.

Mayor, D.J., Sommer, U., Cook, K.B. & Viant, M.R. (2015). The metabolic response of marine copepods to environmental warming and ocean acidification in the absence of food. *Sci. Rep.* , 5, 13690.

Mitchell, M.D., Bairos-Novak, K.R. & Ferrari, M.C.O. (2017). Mechanisms underlying the control of responses to predator odours in aquatic prey.*J. Exp. Biol.* , 220, 1937–1946.

Nicholson, J.K., Connelly, J., Lindon, J.C. & Holmes, E. (2002). Metabonomics: A platform for studying drug toxicity and gene function.*Nat. Rev. Drug Discov.* , 2, 153–161.

Orsini, L., Spanier, K.I. & De Meester, L. (2012). Genomic signature of natural and anthropogenic stress in wild populations of the waterflea*Daphnia magna* : Validation in space, time and experimental evolution. *Mol. Ecol.* , 21, 2160–2175.

Otte, K.A., Fröhlich, T., Arnold, G.J. & Laforsch, C. (2014). Proteomic analysis of *Daphnia magna* hints at molecular pathways involved in defensive plastic responses. *BMC Genomics* , 15, 306.

Pauwels, K., Stoks, R. & De Meester, L. (2005). Coping with predator stress: Interclonal differences in induction of heat-shock proteins in the water flea *Daphnia magna* . *J. Evol. Biol.* , 18, 867–872.

Pauwels, K., Stoks, R. & De Meester, L. (2010). Enhanced anti-predator defence in the presence of food stress in the water flea *Daphnia magna* . *Funct. Ecol.* , 24, 322–329.

Price, T.D., Qvarnstrom, A. & Irwin, D.E. (2003). The role of phenotypic plasticity in driving genetic evolution. *Proc. R. Soc. B Biol. Sci.* , 270, 1433–1440.

Ratnasekhar, C., Sonane, M., Satish, A. & Mudiam, M.K.R. (2015). Metabolomics reveals the perturbations in the metabolome of*Caenorhabditis elegans* exposed to titanium dioxide nanoparticles.*Nanotoxicology* , 9, 994–1004.

Reznick, D.N., Shaw, F.H., Rodd, F.H. & Shaw, R.G. (1997). Evaluation of the rate of evolution in natural populations of guppies (*Poecilia reticulata*). *Science* , 275, 1934–1937.

Robinson, B.W. (2013). Evolution of growth by genetic accommodation in Icelandic freshwater stickleback. *Proc. R. Soc. B Biol. Sci.* , 280, 20132197.

Schoener, T.W., Losos, J.B., Kolbe, J.J., Lapiedra, O. & Leal, M. (2018). Predator-driven natural selection on risk-taking behavior in anole lizards. *Science* , 360, 1017–1020.

Schwarzenberger, A., Courts, C. & von Elert, E. (2009). Target gene approaches: Gene expression in *Daphnia magna* exposed to predator-borne kairomones or to microcystin-producing and microcystin-free *Microcystis aeruginosa* . *BMC Genomics* , 10, 1–15.

Scoville, A.G. & Pfrender, M.E. (2010). Phenotypic plasticity facilitates recurrent rapid adaptation to introduced predators.*Proc. Natl. Acad. Sci. U. S. A.* , 107, 4260–4263.

Southam, A.D., Weber, R.J.M., Engel, J., Jones, M.R. & Viant, M.R. (2017). A complete workflow for high-resolution spectral-stitching nanoelectrospray direct-infusion mass-spectrometry-based metabolomics and lipidomics. *Nat. Protoc.* , 12, 255–273.

Stoks, R., Govaert, L., Pauwels, K., Jansen, B. & De Meester, L. (2016). Resurrecting complexity: The interplay of plasticity and rapid evolution in the multiple trait response to strong changes in predation pressure in the water flea *Daphnia magna* . *Ecol. Lett.* , 19, 180–190.

Taylor, N.S., Kirwan, J.A., Johnson, C., Yan, N.D., Viant, M.R., Gunn, J.M., *et al.* (2016). Predicting chronic copper and nickel reproductive toxicity to *Daphnia pulex* -pulicaria from whole-animal metabolic profiles. *Environ. Pollut.* , 212, 325–329.

Toyota, K., Gavin, A., Miyagawa, S., Viant, M.R. & Iguchi, T. (2016). Metabolomics reveals an involvement of pantothenate for male production responding to the short-day stimulus in the water flea, *Daphnia pulex* . *Sci. Rep.* , 6, 25125.

Viant, M.R., Kurland, I.J., Jones, M.R. & Dunn, W.B. (2017). How close are we to complete annotation of metabolomes? *Curr. Opin. Chem. Biol.* , 36, 64–69.

Viant, M.R., Rosenblum, E.S. & Tjeerdema, R.S. (2003). NMR-based metabolomics: A powedul approach for characterizing the effects of environmental stressors on organism health. *Environ. Sci. Technol.* , 37, 4982–4989.

Weider, L.J., Jeyasingh, P.D. & Frisch, D. (2018). Evolutionary aspects of resurrection ecology: Progress, scope, and applications—an overview. *Evol. Appl.* , 11, 3–10.

Westerhuis, J.A., Hoefsloot, H.C.J., Smit, S., Vis, D.J., Smilde, A.K., Velzen, E.J.J., *et al.* (2008). Assessment of PLSDA cross validation. *Metabolomics* , 4, 81–89.

Westrick, S.E., Broder, E.D., Reznick, D.N., Ghalambor, C.K. & Angeloni, L. (2019). Rapid evolution and behavioral plasticity following introduction to an environment with reduced predation risk.*Ethology* , 125, 232–240.

Xia, J. & Wishart, D.S. (2011). Web-based inference of biological patterns, functions and pathways from metabolomic data using MetaboAnalyst. *Nat. Protoc.* , 6, 743–760.

Xuan, J., Pan, G., Qiu, Y., Yang, L., Su, M., Liu, Y., *et al.*(2011). Metabolomic profiling to identify potential serum biomarkers for schizophrenia and risperidone action. *J. Proteome Res.* , 10, 5433–5443.

Figure captions

Figure 1 Metabolomic profiling of *Daphnia magna* . PCA score plots for (a) the positive and (b) the negative ion mode of all three subpopulations. PLS-DA score plots for (a, c, e) the positive ion and (b, d, f) the negative ion mode of each subpopulation. LV1 and LV2 are the first two latent variables.

Figure 2 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.

Figure 3 Venn diagram of the significantly up-regulated and down-regulated metabolites in response to fish kairomones for each subpopulation of *Daphnia magna* for the (a) positive and (b) negative ion modes.

Figure 4 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 5 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 S1. Each small black dot represents a single metabolite peak. Large black dots represent the mean values of each component.

Figure 6 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.

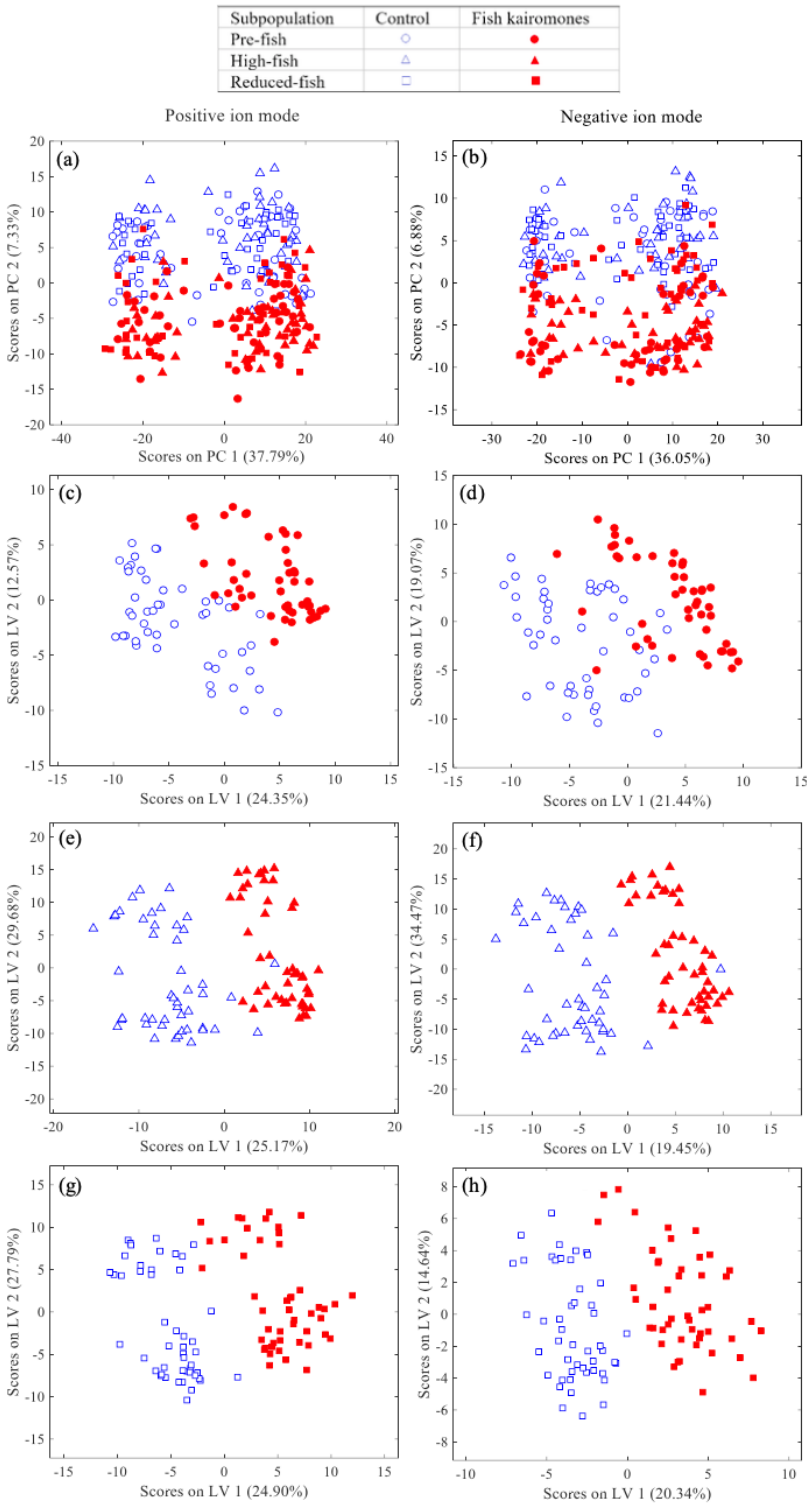

Figure 1

Figure 1

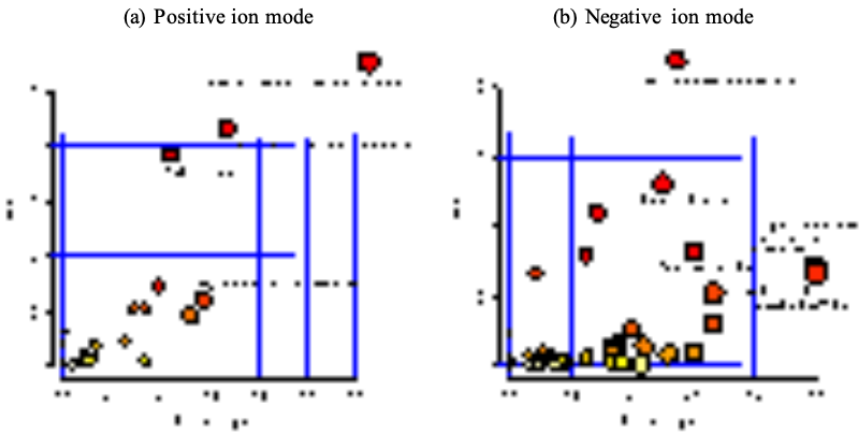

Figure 2

Figure 2

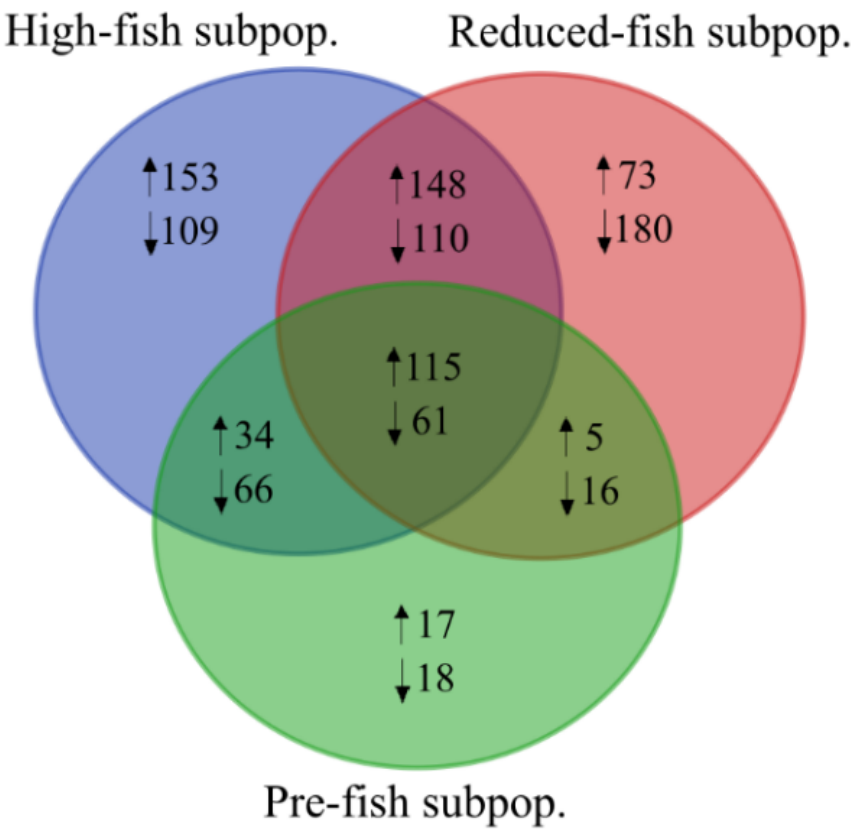

Figure 3

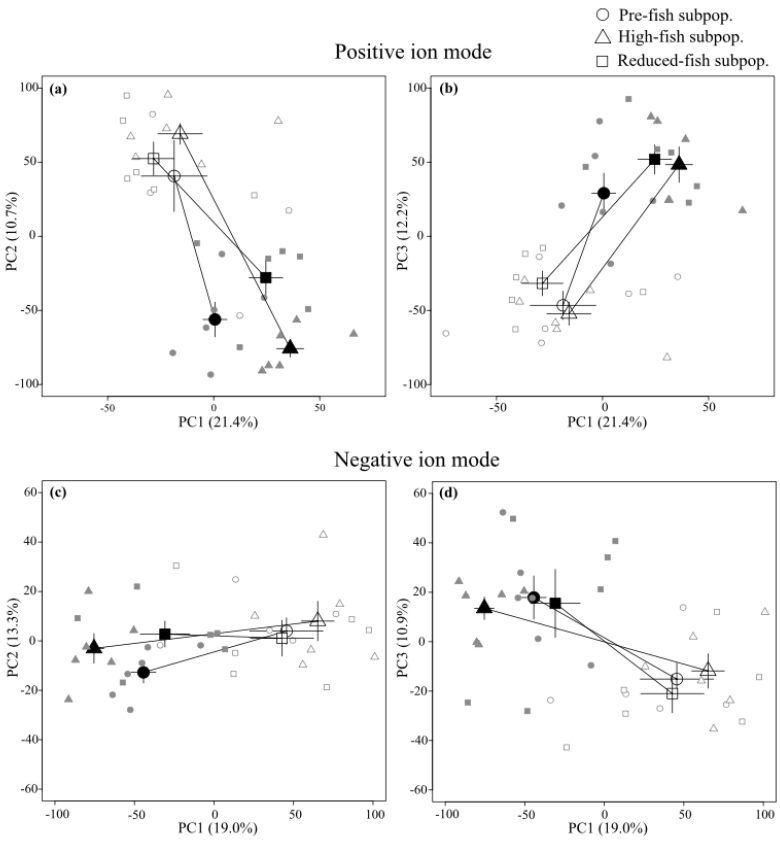

Figure 4

Figure 4

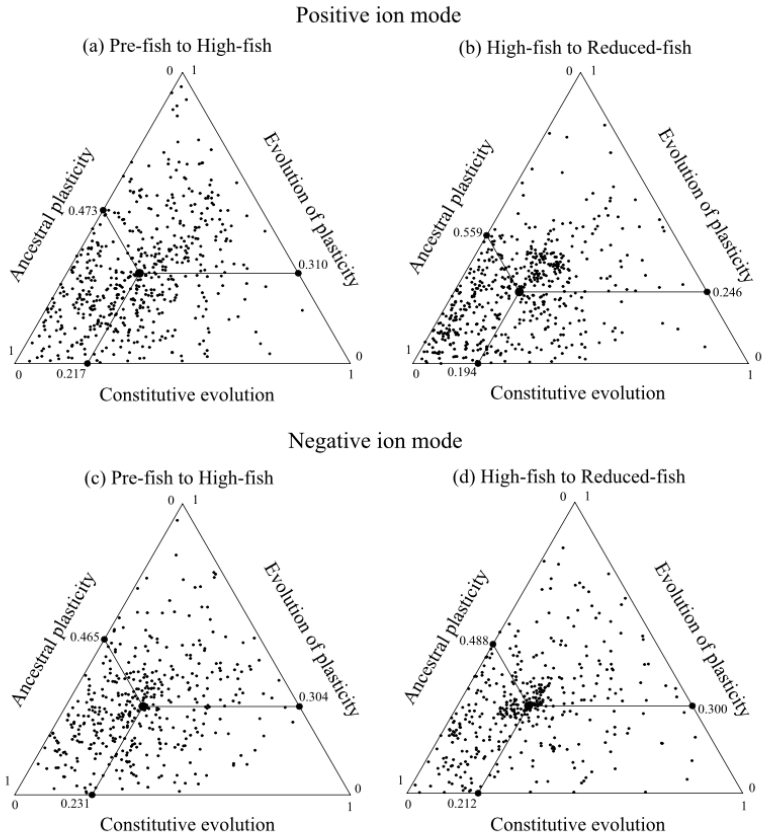

Figure 5

Figure 5

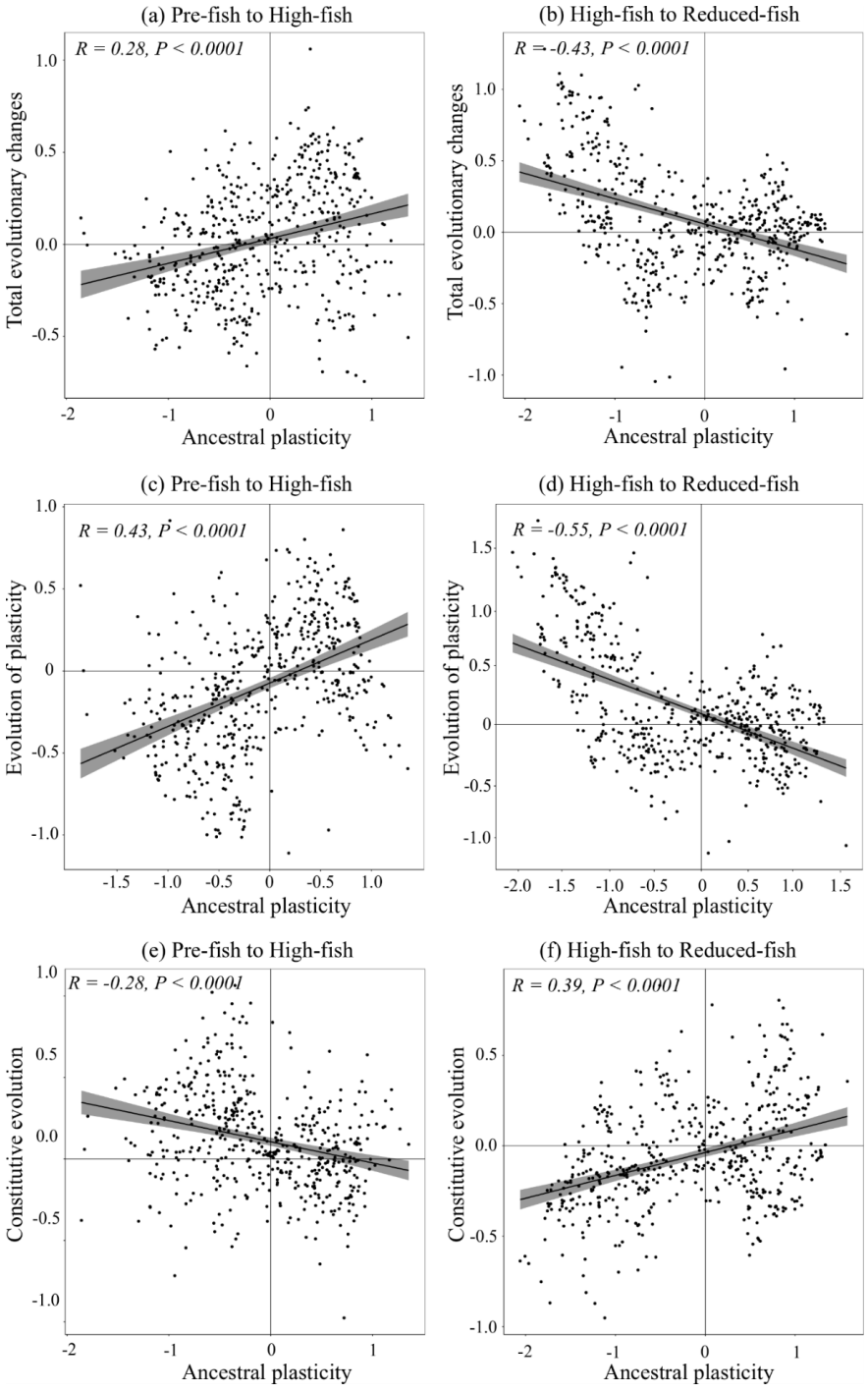

Figure 6