Transcriptomic bases of within- and trans-generational predator-induced plasticity in the freshwater snail *Physa acuta*

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Abstract

Inducible defences in response to predation risk are a well-known example of adaptive phenotypic plasticity. While inducible defences have been mainly studied within a generation (within-generational plasticity), there is now clear evidence that the ancestral exposure to predation risk can influence the defences expressed by the offspring, even if they have not been exposed themselves (transgenerational plasticity). The molecular mechanisms allowing the transmission of environmental information across generations are not well understood. In this study, we combined measures of anti-predator responses (behavioural and morphological) with transcriptomic investigations across two generations in the freshwater snail *Physa acuta*. We hypothesised that both within- and transgenerational plasticity would induce phenotypic changes associated with differential gene expression. Our results confirmed within- and transgenerational plasticity: F1 snails have responded to predator-cue exposure by increasing escape behaviour, reducing shell length, and developing thicker and slender shells and F2 from exposed parents have longer and thicker shells with a narrower aperture. Within- and transgenerational plasticity were accompanied by the differential expression of 112 genes (101 up- and 11 down-regulated) and 23 differentially expressed genes (17 up- and 6 down-regulated), respectively. Within- and transgenerational plasticity did not share common differentially expressed genes but the molecular functions were similar, involving metabolism and transcription regulation. These results suggest that

predator-induced WGP and TGP may result from different genomic pathways and may evolve independently.

INTRODUCTION

Phenotypic plasticity is the ability of a genotype to produce alternative phenotypes under different environmental conditions (Pigliucci, 2005). Plastic responses are widespread across the phylogenetic spectrum (*e.g*. Galloway & Etterson, 2007; Luquet et al., 2011; Segers & Taborsky, 2011; Walsh et al., 2016), and are a major concern in evolutionary biology as they can be adaptive, i.e. increasing fitness of organisms facing environmental changes (Pigliucci, 2005). Phenotypic plasticity classically refers to within-generational plasticity (WGP), meaning that it occurs within one generation. Recently, plasticity has also been found to occur across generations (transgenerational plasticity, TGP) where the phenotype of an organism is influenced by the environment experienced by the previous generations (Bell & Hellmann, 2019). Interest in TGP has grown rapidly in recent years with evidence that TGP is at least as widespread as WGP (Jablonka & Raz, 2009; Tariel, Plénet, et al., 2020b). Although there are numerous examples of TGP generating seemingly adaptive offspring phenotypes, its adaptive role is expected to be more challenging than that of WGP due to patterns of environmental variation across generations (Colicchio & Herman, 2020). Indeed, it requires a strong temporal autocorrelation between environments

76 of distinct generations to be selected (Ezard et al., 2014; Kuijper & Hoyle,
77 2015; Prizak et al., 2014) and a transmission system that allows the induction
78 of adaptive phenotypes across generations (Fallet et al., 2020). The
79 inheritance mechanisms appear diverse, from epigenetic changes to
80 parental transmission of cytoplasmic elements (small RNAs, nutrients,
81 hormones, proteins), parental care and cultural transmission (Bell &
82 Hellmann, 2019; Danchin et al., 2011).

83 Regardless of the transmission mechanisms, these should lead in the
84 offspring to the modification of the expression of some of the genes involved
85 in the phenotypes, at least in some tissues and at some stages during the
86 development. Surprisingly, transgenerational plastic response at the
87 molecular level is overlooked. This is indeed a necessary first step to unravel
88 the molecular mechanisms of plasticity in general and their transgenerational
89 inheritance. The most compelling explorations of changes in gene
90 expression profiles in response to both developmental (WGP) and parental
91 (TGP) environments come from the studies of Hales et al. (2017) on *Daphnia*
92 *ambigua* and Stein et al. (2018) on three spined stickleback (*Gasterosteus*
93 *aculeatus*). These studies compared gene expression between individuals
94 that had been exposed to predator cues relative to non-exposed individuals
95 within a generation (WGP genes) and among their offspring (from non-
96 exposed and exposed parents; TGP genes). Stein et al. (2018) found that
97 the same group of genes changed their expression in offspring whether the
98 predation risk was experienced by the offspring (TGP), the father (WGP), or
99 both. This suggests that a core set of genes is consistently affected by

100 predation risk regardless of who experiences it. On the other hand, Hales et
101 al. (2017) found that changes in the expression of genes that respond to TGP
102 differed depending on whether exposure to predation was experienced by
103 parents or grandparents. They also showed that very few genes were
104 common to both WGP and TGP. Note that Clark et al. (2019) also showed
105 that TGP gene expression profiles of larvae of sea urchin (*Psammechinus*
106 *miliaris*) spawned at low pH from pre-acclimated adults differed from those
107 of larvae produced by adults exposed to ambient pH, but the authors did not
108 explore the WGP genes. These few results highlight the necessity to
109 investigate together WGP and TGP at the molecular level. Beyond the
110 inheritance mechanisms allowing organisms to memorise past
111 environmental conditions to adjust their future phenotype, we need to know
112 whether the two forms of plasticity are distinct at the molecular level and
113 potentially free to evolve independently of one another (Bell & Stein, 2017).

114 Predation risk is a major selection pressure for most species and results in
115 the evolution of defences in prey (Kikuchi et al., 2023). As predation risk often
116 varies over time and space, it favours the evolution of phenotypic plasticity
117 in defences (Reger et al., 2018; Tariel, Plénet, et al., 2020b; Viney & Diaz,
118 2012). Indeed, while some defences against predators are constitutive,
119 plastic defences are only induced when prey detect some predator cues in
120 their environment (*e.g*. visual cues, chemical cues such as predator odours,
121 or alarm signals released by conspecifics). These induced defences are
122 classic examples of adaptive WGP: prey can increase their fitness by
123 producing defences only when predators are present. The links between

124 prey-predator interactions and WGP have been studied for a long time (*e.g.*
125 Tariel et al., 2020b; Tollrian, 1995). Plastic defences can also be induced in
126 offspring after their parents or more distant ancestors have detected predator
127 cues. This transgenerational induction of defences was first highlighted in
128 Daphnia morphology by Agrawal et al. (1999) and has since been repeatedly
129 shown in multiple species and defences (review in MacLeod et al., 2022;
130 Tariel, Plénet, et al., 2020b).

131 Consequently, this work aims to unravel the molecular mechanisms
132 underlying the expression of WGP and TGP of defences in the context of
133 predator-prey interactions. We used *Physa acuta* (also referred to as
134 *Physella acuta*), a freshwater snail known for their within- and
135 transgenerational anti-predator defences such as escape behaviour (snails
136 crawl out of the water when they or their parents detect predator cues;
137 (Luquet & Tariel, 2016; Tariel, Luquet, et al., 2020; Tariel, Plénet, et al.,
138 2020a; Turner et al., 1999) change in shell size and shape, increase in shell
139 thickness and overall increase in shell-crush resistance (*e.g.* shell harder to
140 handle or to crush by predators; (Auld & Relyea, 2011; Beaty et al., 2016;
141 DeWitt, 1998; Tariel, Plénet, et al., 2020c; Tariel-Adam et al., 2023). We
142 combined a two-generation experiment to generate predator-induced WGP
143 and then TGP of defences (only in a non-exposed environment) with a
144 transcriptomic investigation. We used transcriptome sequencing and
145 assembly to specifically investigate the transcriptome mantle skirt gene
146 expression (mantle skirt is the tissue that synthesises the shell). We
147 expected that gene expression should be affected by current (WGP) and

148 parental (TGP) exposure to predator cues. More specifically, as WGP and
149 TGP induce similar phenotypic responses (*e.g*. increase in shell thickness,
150 we expected the two processes share the same core set of genes.

151 **MATERIALS & METHODS**

152

153 **Animal collection, rearing conditions and experimental design**

154 Adult *P. acuta* snails were collected from a wild population in a lentic
155 backwater of the Rhône River (Lyon, France, 45° 48'6"N, 4° 55'33"E) in
156 September 2018. Individuals from this population have been repeatedly used
157 in previous studies on predator-induced plasticity (Luquet & Tariel, 2016) and
158 are naturally exposed to predation by the crayfish *Faxonius limosus*. The
159 experiment was conducted in our laboratory in a temperature-controlled
160 chamber at 25 °C with a photoperiod of 12/12h. The wild snails constituting
161 the F0 generation were pooled in a 10 L plastic box filled with dechlorinated
162 tap water (control water hereafter) and interbreed overnight. They were then
163 isolated in 80 mL plastic boxes (4.5 x 6 cm) filled with tap control (*i.e.*, without
164 predator cues) water to lay eggs for 3 days. Eggs within boxes constitute F1
165 matrilines. Eggs hatched seven days later and then siblings grew together
166 for a further ten days in water without predator cues. Then, 10 siblings of 20
167 F1 matrilines were randomly selected and split into two developmental
168 environments: five siblings in water without predator cues (non-exposed
169 treatment) and five siblings in water with predator cues (exposed treatment).
170 F1 siblings individually developed for the next 31 days in 80 mL plastic boxes.
171 On this date, one F1 snail per matriline and treatment was frozen at -80°C
172 for molecular analysis and all remaining F1 snails were measured (see
173 below).

174 To generate the F2 generation, we pooled F1 snails from the same treatment
175 in an aquarium filled with control water and let them copulate for 24h. As
176 previously, F1 snails were then isolated in 80 mL boxes and laid eggs for 3
177 days to produce the F2 matrilines. F2 snails from the two parental
178 environments (non-exposed and exposed to predator cues) were only reared
179 in control water in order to investigate the effect of parental environment
180 (TGP) alone, i.e. without the interaction between parental and developmental
181 environments. The same protocol steps as previously for F1 snails were
182 followed, except that eight siblings per matriline were reared and
183 measurements were made at an older age because of a slower growth. As
184 for F1, one F2 snail per matriline and treatment was frozen for molecular
185 analysis and the others were measured.

186 Snails have been fed *ad libitum* with boiled and mixed lettuce. Water
187 (according to treatment) and food was renewed twice a week. The water with
188 predator cues (i.e. exposed treatment) was the was water in which *F. limosus*
189 crayfish have been raised (one crayfish/5L), for 3 days and fed with snails.
190 Additionally, to ensure fresh alarm cues, we smashed several *P. acuta* adult
191 snails in the predator-cue water (one snail/5L) for 1h before using.

192

193 **Behavioural and morphological responses**

194 In this study, in order to check that exposure to predator cues triggered WGP
195 and TGP, escape behaviour and shell morphology were measured on F1
196 (non-exposed snails = 76, exposed snails = 73) and F2 (snails from non-

197 exposed parents = 89, snails from exposed parents = 101). A few mortalities
198 have led to uneven sample sizes but the mortality rate was consistent across
199 families (see Table S1). Crawling out of the water (position above the water
200 surface) is considered as allowing snails to escape from benthic predators
201 such as crayfishes (DeWitt, 1998; Tariel, Plénet, et al., 2020a). Shorter and
202 narrower shell and aperture dimensions and thicker shell are adaptive anti-
203 predator responses (Auld & Relyea, 2011). These phenotypic traits have
204 been recurrently involved in transgenerational responses of *P. acuta* to
205 predator cues (Beaty et al., 2016; Luquet & Tariel, 2016).

206 *Escape behaviour* - One week before measurements and four hours after the
207 water renewal, we recorded the position above or below the water surface of
208 each snail in their rearing boxes. Consequently, the behaviour of F1 snails
209 was recorded in water without or with predator cues according to their
210 respective treatments while that of F2 snails was only recorded in water
211 without predator cues.

212 *Shell thickness, shell length and shape* - Shell thickness was directly
213 measured with an electronic calliper at the nearest 0.01 mm at the edge of
214 the aperture. After, a photograph of each snail (aperture upwards) was taken
215 with an Olympus SC50 camera installed on an Olympus SZX9 binocular and
216 its Olympus DF PLAPO 1X-2 objective at a x8 magnification. Snail shell
217 length, shell width, aperture length and aperture width were measured using
218 ImageJ (Schneider et al., 2012). To estimate potential changes in shell shape
219 according to treatment, we used geometric methods (Gustafson et al., 2014).
220 On each snail shell, we digitised 19 landmarks (Fig. 1) using the software

ImageJ. Snails that lacked homologous landmarks because of broken shells (typically broken apertures and apexes) or small size (lack of the third shell spire) were removed from the analyses (non-exposed F1 snails = 76, exposed F1 snails = 72, F2 snails from non-exposed parents = 89, F2 snails from exposed parents = 99). To compare shell shapes according to treatments, we scaled the shapes using the size metric defined below (Eqn. 1). Translational components were removed by translating all shapes so that the centroids lie at the origin. Rotational components were removed by rotating all shapes until the Procrustes distance was minimised with the first shell fixed as reference.

$$s_k = \sqrt{\sum_{i=1}^{19} ((x_{k,i} - \bar{x}_k)^2 + (y_{k,i} - \bar{y}_k{}^2)}$$

Equation 1: Procuste's equation used to scale the shapes using the size metric s_k is the size of the k-th shell, $x_{k,i}$ and $y_{k,i}$is the x and y component of the i-th landmark, and $(\bar{x}_k, \bar{y}_k)$, is the centroid of the k-th shell.

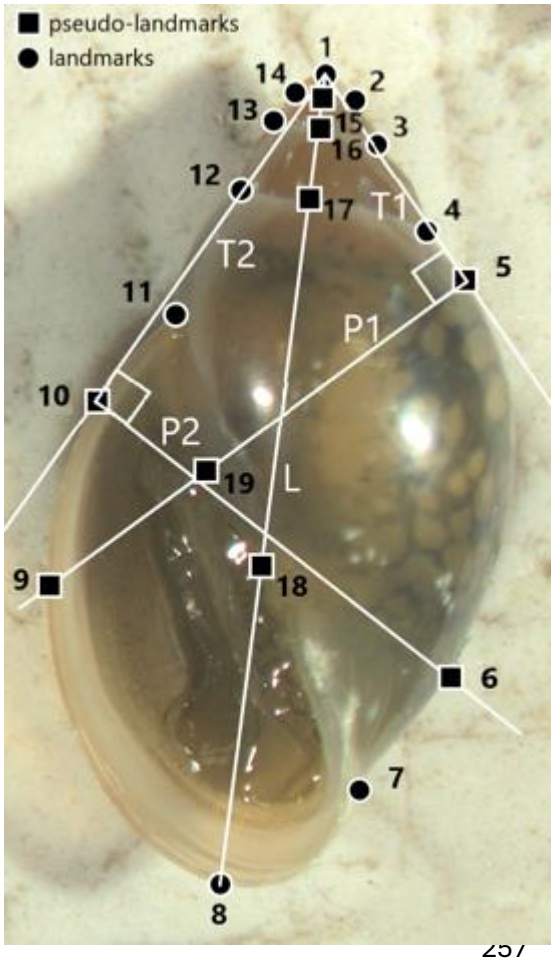


Figure 1: Locations of the different landmarks (circle symbol) and pseudo-landmarks (positioned using a geometric method, square symbol) on *P. acuta* shell. Pseudo-landmarks have been created using a geometric method: first, two tangents (T1 and T2) have been drawn from landmark 1 (apex). T1 touches the curve of the habitation spire (5) and T2 touches the external edge of the aperture (10). From the two points of tangency (points 5 and 10), two perpendicular lines to the tangents have been traced (P1 and P2). The intersections with the columellar edge of the aperture, the external edge of the aperture and the curve of the habitation spire give additional pseudo-landmarks (6, 9, 18 and 19). The length of the shell L (straight line passing by 1 and 8) gives the pseudo-landmark 18 at the intersection with the columellar edge of the aperture).

Statistical analyses

The same statistical analyses were separately performed to test for WGP on F1 snails and TGP on F2. The treatment factor (i.e. non-exposed or exposed to predator cues) was the developmental environment for WGP (F1) while it was the parental one for TGP (F2).

265 *Escape behaviour* - The effects of predator cues on escape behaviour (i.e.
266 snail position above/on or below the water surface) were analysed using
267 generalised linear mixed models (GLMM) assuming a binomial distribution
268 (logit link function). The treatment was a fixed effect and matriline was a
269 random intercept effect. We tested significance of fixed and random effects
270 with likelihood ratio tests.

271 *Shell thickness, shell length* - To analyse the effects of predator cues on shell
272 length and shell thickness, we performed linear mixed models with treatment
273 as fixed effect and matriline as a random intercept. We used restricted
274 maximum likelihood estimation and Kenward and Roger's approximation for
275 degrees of freedom. We tested significance of fixed effects with type II F-
276 tests (Kuznetsova et al., 2017) and significance of random effect with
277 likelihood ratio tests.

278 Linear mixed models and generalised linear mixed models were performed
279 using the lme4 package (Bates et al., 2014) on R software (version R-4.4.1
280 for Windows, (RStudio Team, 2020).

281 *Shell shape* - The effects of predator cues on shell shape were analysed
282 using principal component analyses (PCA) and were visualised with the 2D
283 landmarks relative warps analysis functionality. All standardised shapes
284 obtained by Procustes method were analysed using the PAST software with
285 a Student test (Hammer et al., 2001).

286

287 **Molecular biology analysis**

288 The mantle skirt was dissected for 5 non-exposed F1 snails and 5 exposed
289 F1 snails, and for 10 F2 snails from non-exposed parents and 10 F2 from
290 exposed parents. Total RNA of the mantle was extracted by adding TRI
291 Reagent (Molecular Research Center MRC, TR118) to the samples and by
292 homogenising tissues on ice with pistons and agitator (Motor Mixer Clearline
293 ref.045011 Dutscher). All remaining steps were carried out according to the
294 manufacturer's protocols (Molecular Research Center MRC, TR118). Then,
295 the RNA was treated with Turbo DNase enzyme (Turbo DNA free kit,
296 Invitrogen, AM1907) and assayed by fluorescence using a Qubit fluorometer
297 (Qubit® RNA HS Assay Kits Molecular Probes, Invitrogen, Q32855). Library
298 construction was carried out using the NEBNext Ultra II RNA Library Prep Kit
299 for Illumina (Biolabs New England, NEB E7770, E7490, E7600). Libraries
300 were randomly split in three groups and sequenced on three lanes with an
301 Illumina® HiSeq4000™ machine on the GenomEast platform hosted at the
302 IGBMC (GenomEast Platform – Institut de Génétique et de la Biologie
303 Moléculaire et Cellulaire IGBMC, Illkirch, France), resulting in 50 bp long
304 single-end reads.

305

306 **Differential gene expression analysis**

307 FASTQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/)) was
308 used to assess the quality of the reads. We used a reference genome ORF
309 *P. acuta* available on NCBI (Isolate: Inbredline_101_S28. RefSeq:
310 GCF_028476545.12). Gene expression levels were assessed on annotated

311 transcripts using Kallisto (-l 50 -s 10 options; Bray et al., 2016). We
312 aggregated effective counts at the gene level and then applied a rounding to
313 the nearest whole number to convert non-integer values into integer counts.
314 We also applied a minimum count filter of 1 (cpm) where we removed all the
315 genes with reads below 1 (i.e. removing 5634 genes from the total of 46965).
316 The rounded effective counts was used as input for differential gene
317 expression using the DESeq2 package (Love et al., 2014), which performs
318 differential gene expression analysis using negative binomial distribution and
319 shrinkage estimation for dispersions ("apeglm" type shrinkage, Zhu et al.
320 2019), and outputs expression fold changes to improve the interpretability of
321 estimates. We specified non-exposed or exposed treatment in the model
322 (design formula: "design = ~treatment") within each generation
323 independently, meaning we compared F1 snails from non-exposed and
324 exposed treatments, and F2 snails from non-exposed and exposed parents.
325 Firstly, the Wald test (DESeq2 package) was used to test the significance of
326 differentially expressed genes (DEGs) in all samples between the non-
327 exposed and exposed treatments. Secondly, we checked for any structures
328 in the normalised count table by performing a principal component analysis
329 (PCA) in all samples on the 500 most DEGs from DESeq2 analysis (pval <
330 0.05) and based on Log2FoldChange (|LFC|, i.e. the logarithm base 2 of the
331 fold change in gene expression between two experimental conditions,
332 providing a measure of both the magnitude and direction of differential
333 expression). Thirdly, we filtered the significant DEGs by a |LFC| greater than
334 1 in each generation. Consequently, only differential expression with

335 an adjusted p-value < 0.05 and |LFC| > 1 was considered thereafter. Then,
336 we investigated whether the DEGs in F1 were also differentially expressed
337 in F2 by comparing specific gene IDs across the two generations datasets.
338 Finally, as the quality of annotations of the reference genome did not allow
339 complex functional analyses, we determined the potential gene functions and
340 the associated protein families of all significant DEGs by using the scan
341 procedure of InterPro, a tool provided by the European Bioinformatics
342 Institute from the European Molecular Biology Laboratory (EMBL-EBI; (Blum
343 et al., 2024; Jones et al., 2014). InterPro integrates signatures from 13
344 member databases to classify proteins into families, to predict functional
345 domains, and to identify key sites, enabling comprehensive functional
346 annotation.

347

348 **RESULTS**

349

350 **Predator-induced behaviour and shell morphology**

351 *Within-generational Plasticity*

352 In F1 generation, the proportion of crawling-out behaviour increased
353 significantly in snails exposed to predator-cues (+85.25%, $F[1,19.43] =$
354 321.82, $p < 0.001$; Fig. 2A). Shell length significantly decreased in snails
355 exposed to predator cues (-6.3%, $F[1,128.71] = 15.46$, $p < 0.001$; Fig. 2B).
356 Shell thickness significantly increased in exposed snails (+18.8%,

F[1,129.58] = 4.02, p = 0.047; Fig. 2C). The matriline random effect was significant for shell length ($\chi^2[1] = 19.022$, $p < 0.001$) and non-significant for behaviour and shell thickness ($\chi^2[1] = 0.690$, $p = 0.406$ and $\chi^2[1] = 2.6116$, $p = 0.106$, respectively).

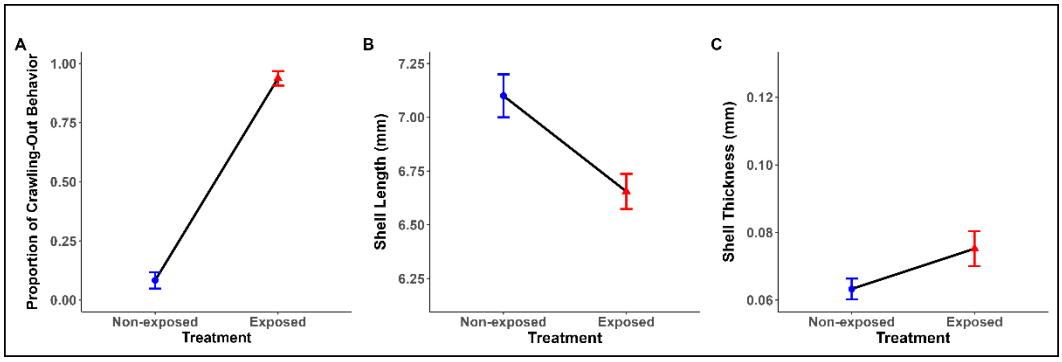


Figure 2: Effect of developmental exposure to predator cues (WGP) on crawling-out behaviour, shell length and shell thickness in the F1 generation. A: Proportion of Crawling-out behaviour; B: Mean shell length (mm); C: Mean shell thickness (mm). Error bars represent SE. Non-exposed and exposed to predator-cue treatments are symbolised with blue circles and red triangles, respectively.

Predator-cues exposure induced changes in shell shape. The two first axes of PCA analysis accounted for 59.2% of the shell shape variation (Fig. 3) and PCA axes 1-4 (74.2% cumulative shape variation) were the only axes to each account for more than 5% of shape variation. PC1 showed a change in the columellar area (close to landmark 18-19) from shells with relatively wide aperture (dilated columellar area; Fig. 3C) to shells with relatively narrow aperture (compressed columellar area; Fig. 3D). PC2 showed a change in the slenderness of the shell from shells with relatively short spires

(compressed apex; Fig. 3B) and relatively wide apertures (dilated aperture) to shells with relatively short spires (dilated apex) and narrow aperture widths (compressed aperture; Fig.3A).

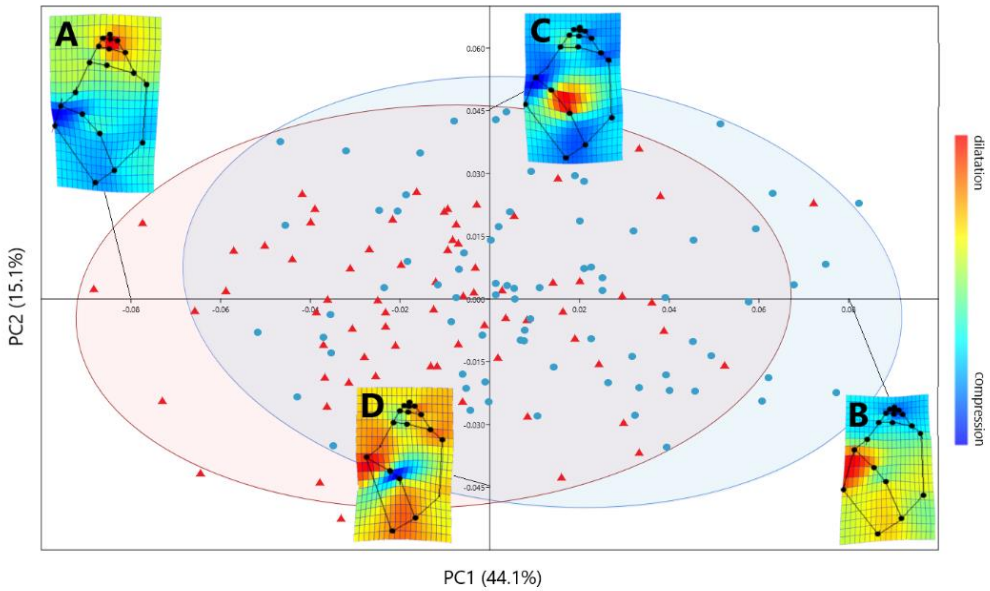


Figure 3: Shape variation of non-exposed and exposed snails from F1 generation (blue circle and red triangle symbols, respectively. Panels A, B, C, D represent deformation grids showing shell shape on the extreme ends of the PCA axes. The 95% confidence ellipses are represented for non-exposed and exposed to predator-cue treatments. The dilation-compression gradient bar shows the deformation of the shell landmarks relative to the points of an average shell (which would be located at the centre of the PCA).

Non-exposed snails showed the same range of deformation of columellar area than exposed snails (PC2, t-test: t = 1.07, df = 144, p = 0.28). However, shells of snails exposed to predator cues are slender, with longer spires and narrow aperture widths than non-exposed ones (PC1, t-test: t = 4.803, df =

144, $p < 0.001$). This elongation is associated with the dilatation of the three last spires and a narrow aperture at the base of the external edge. In contrast, shells of non-exposed snails have a wider aperture at the base of the external edge and smaller spires.

Transgenerational plasticity

In F2 generation, the proportion of crawling-out did not differ between snails from non-exposed and exposed parents ($F[1,20.31] = 3.929$, $p = 0.061$; Fig. 4A). Snails from parents exposed to predator cues were significantly longer and had a thicker shell than those from non-exposed parents (+4.9%, $F[1,170.87] = 7.55$, $p = 0.006$ and +16.9%, $F[1,174.11] = 9.858$, $p = 0.002$, respectively; Fig. 4B-C).-The matriline random effect was significant for shell length ($\chi^2[1] = 31.685$, $p < 0.001$) and non-significant for behaviour and shell thickness ($\chi^2[1] = 0.022$, $p = 0.882$ and $\chi^2[1] = 1.882$, $p = 0.17$, respectively).

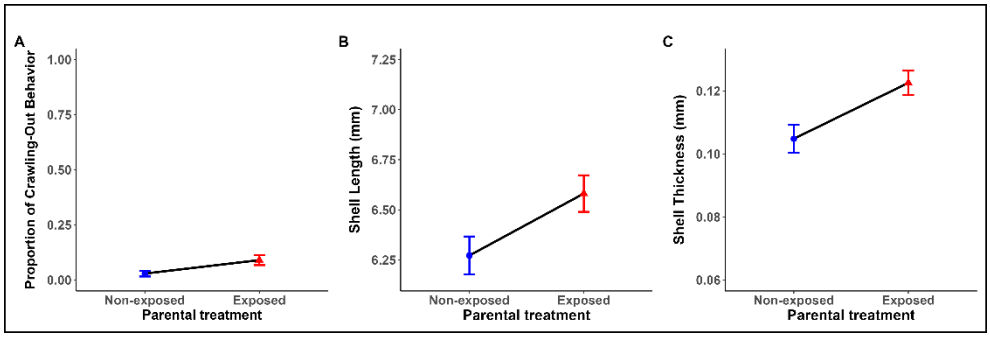


Figure 4: Effect of parental exposure (TGP) on crawling-out behaviour, shell length and shell thickness in the F2 generation. A: Proportion of Crawling-out behaviour; B: Mean shell length (mm); C: Mean shell

thickness (mm). Error bars represent SE. Non-exposed and exposed to predator cues treatments are symbolised with blue circles and red triangles, respectively.

Parental exposure to predator cues induced changes in shell shape. The two first axes of PCA analysis accounted for 63.8% of the shell shape variation and cumulative accounting for PCA axes 1-4 is 80.14% (Fig. 5). PC1 displayed a change in shell aperture width; the shells with high PC1 scores (Fig. 5C) showed a dilatation of the shell where the outer lip meets the parietal wall and a small compression of the columellar area (i.e. a wider aperture). Shells with low PC1 scores (Fig. 5D) presented a narrower aperture, a consequence of a dilated columellar area but compressed parietal wall and basal lip in comparison to shells with high PC1 score. PC2 showed a change in shell aperture width but also in shell length, from shells (Fig. 5C) with a dilated outer lip and a compression of the apex (which tends to reduce spire length i.e. overall shell length) to shells (Fig. 5D) showing elongated spires from dilated apex, with a narrower aperture due to outer lip compression.

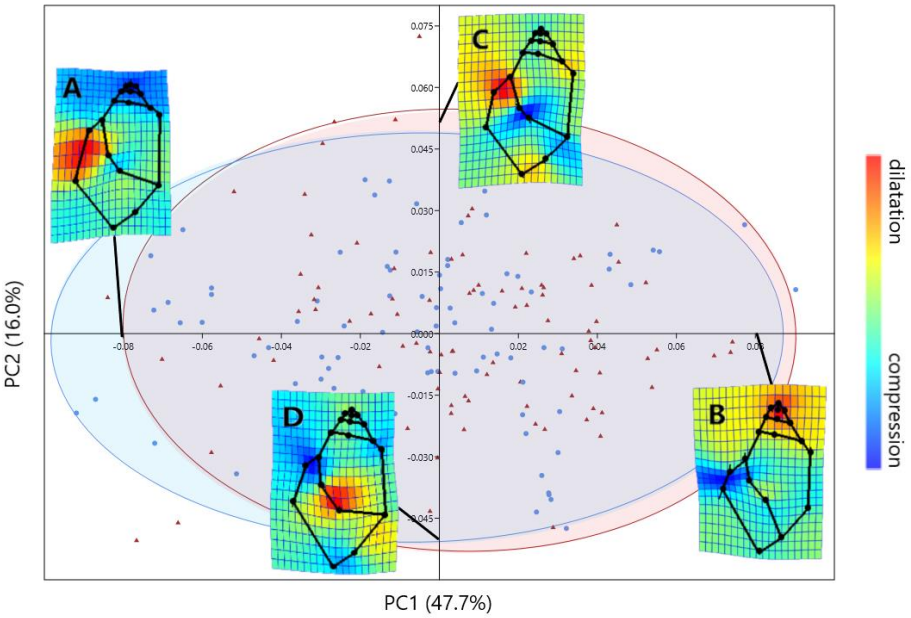
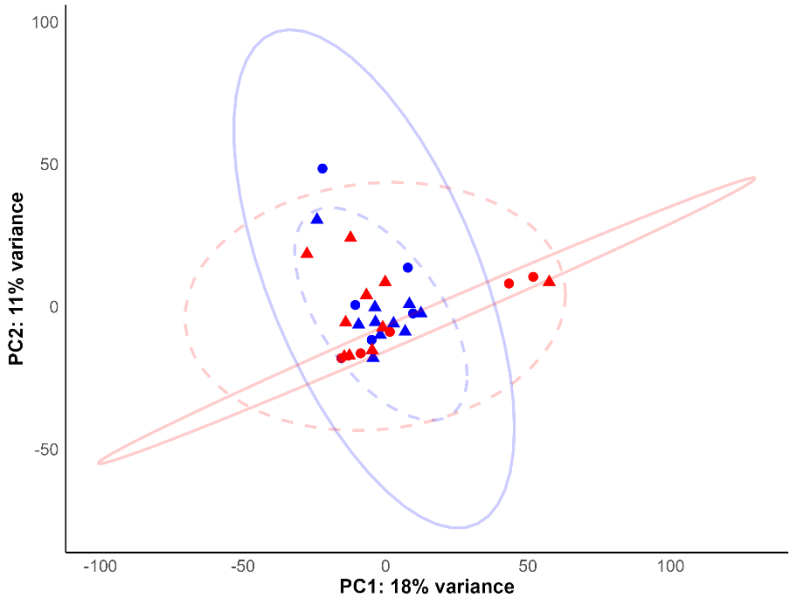


Figure 5: Shape variation of F2 snails from non-exposed and exposed parents (blue circle and red triangle symbols, respectively). Panels A, B, C, D represent deformation grids showing shell shape on the extreme ends of the PCA axes. The 95% confidence ellipses are represented for non-exposed and exposed to predator-cue treatments. The dilation-compression gradient bar shows the deformation of the shell landmarks relative to the points of an average shell (which would be located at the centre of the PCA).

Snails from predator-exposed parents had a dilatation of the shell in the columellar area accompanied by a small compression of the parietal wall (PC1: $t = 2.067$, $p = 0.04$). Conversely, there is no difference in deformation of the outer lip, nor dilatation or compression of the apex between snails from exposed and non-exposed parental environments (PC2: $t = 0.59$, $p = 0.55$).

Differential gene expression induced by predator-cues



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444 **Figure 6:** PCA plot of the 30 transcriptome samples of *P. acuta* snails showing the divergence in
445 differential expression among the first 500 most differentially expressed genes (selected by greatest
446 |LFC|). Non-exposed and exposed to predator cue individuals are represented in blue and red,
447 respectively. F1 individuals are represented by circles and plain ellipses and F2 individuals by triangles
448 and dotted ellipses.

449

450 A principal component analysis (PCA) was performed to explore the
451 clustering of read counts on the first 500 most DEGs. The first and second
452 principal components (PC1 and 2) explained 18% and 11% of the variance
453 respectively (Fig. 6, PCA loadings available in Fig. S1). The plot did not show
454 clustering neither between the non-exposed and exposed treatments nor
455 between the F1 and F2 generations (Fig. 6). In the F1 generation, the
456 differential expression analysis revealed a total of 112 DEGs between non-
457 exposed and exposed snails (101 up-regulated genes and 11 down-
458 regulated gene; Fig. S2). In the F2 generation, the differential expression

analysis showed a total of 23 DEGs between snails from non-exposed and exposed parents (17 up-regulated and 6 down-regulated gene; Fig. S2). The comparison of DEGs in F1 and F2 revealed that they were not overlapped (Fig. 7; FigS3).

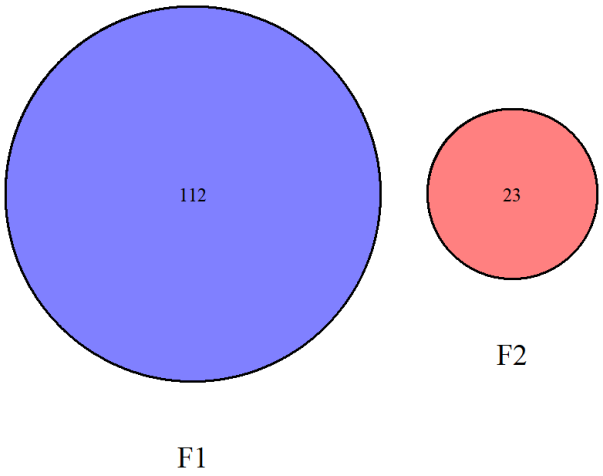
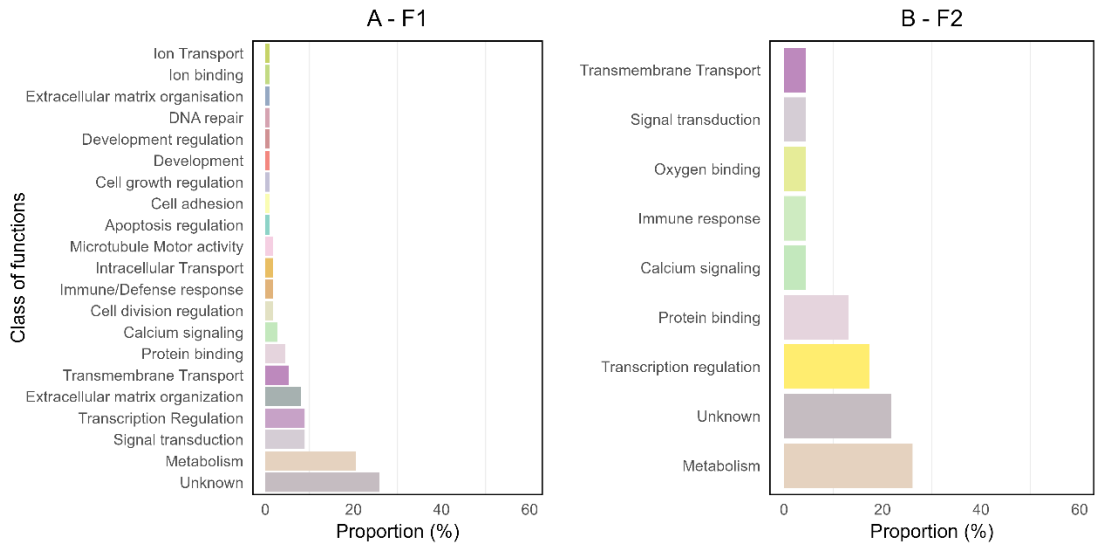


Figure 7: Venn diagram (obtained with DESeq2 package) illustrating the number and overlap of differentially expressed genes between F1 and F2 groups. Genes specific to each group are shown in individual circles, while the intersection represents the shared genes.

The DEGs in F1 are mainly involved in metabolism, signal transduction, extracellular matrix organization, protein and transcriptional regulations (Fig. 8, Tables S2 and S3 for further details). The DEGs in F2 are mainly involved in metabolism and transcriptional regulation, including DNA methylation regulation (Fig. 8, Tables S2 and S3 for further details). In both F1 and F2,

474 some DEGs were associated with calcium signalling (Fig. 8, Tables S2 and
475 S3 for further details) and a main proportion of DEGs was genes with
476 unknown functions.

477



478

479 **Figure 8:** Proportions of classes of functions of the differentially expressed genes in F1 (A) and in F2 (B)
480 based on the InterPro EMBL-EBI database (Jones et al., 2014; Blum et al., 2024).

481

482 **DISCUSSION**

483

484 This work aimed to unravel the molecular mechanisms underlying the
485 expression of within- and trans-generational plasticity of anti-predator
486 defences in *P. acuta*. We expected that gene expression should be affected
487 by developmental (WGP) and parental (TGP) exposure to predator cues, and
488 that both WGP and TGP share the same core set of genes. Exposure to

489 predator cues results in changes in escape behaviour, shell growth, shell
490 thickness and shell shape. The parental exposure to predator cues (TGP)
491 also influenced the offspring phenotype, in particular morphological traits as
492 previously shown in this system (*e.g.* Luquet & Tariel 2016). Accordingly,
493 there were DEGs between non-exposed and exposed snails in F1 (WGP)
494 and between F2 snails according to parental exposure to predator cues
495 (TGP). However, the number of DEGs detected for TGP were lower than for
496 WGP. Finally, our results showed that DEGs for WGP and TGP did not
497 overlap.

498

499 **Phenotypic changes and gene expression induced by within-**
500 **generational exposure to predator cues**

501

502 As expected, snails exposed to predator cues crawled more out of the water,
503 had thicker shells, were smaller and developed slender shells with longer
504 spires and narrower aperture width than non-exposed snails. These
505 behavioural and morphological responses represent defences and are
506 supposed to be adaptive as they are consistent with an escape behaviour
507 (Tariel, Luquet, et al., 2020) and an increase in shell crush resistance (Tariel,
508 Plénet, et al., 2020a, 2020c), increasing snail survival in face of crayfish
509 predation (Auld & Relyea, 2011). The decreased shell size observed in
510 exposed snails may result from a physiological stress caused by exposure

to predator cues, including costs to produce anti-predator defences (*e.g*. crawling-out behaviour is at the expense of foraging; DeWitt, 1998).

These predator-induced WGP was associated with a distinct pattern of gene expression with 112 DEGs (101 up-regulated and 11 down-regulated genes). Overall, this pattern is consistent with several studies that explored the changes in gene expression induced by the environment (*e.g*. Aubin-Horth & Renn, 2009; Herman & Sultan, 2011). In the context of predator-prey interaction, the main knowledge about the transcriptomic responses of preys to predators comes from studies on Daphnia species. For example, Hales et al. (2017) in *Daphnia ambigua* and Rozenberg et al. (2015) in *Daphnia pulex* showed that anti-predator defences, mainly morphological traits, were associated with 48 and 230 DEGs respectively. On the contrary, no DEGs were identified in *Daphnia galeata,* a species known to adjust life history traits (such as reproduction) but not morphological ones in response to predator cues. Similarly, Orsini et al. (2018) revealed no changes in gene expression associated with a short-term exposure to fish kairomones in *Daphnia magna*. These contrasted results suggest that the patterns of gene expression may depend on several factors such as the traits considered and the strength of predator signals (*e.g*. cue concentration, duration of exposure).

The functional analysis of DEGs in snails exposed to predator cues suggested that these genes would be mainly linked with metabolism functions and transcription regulation, both processes known to be intimately associated with stress responses (note however that 26% of DEGs had

534 unknown functions). A major part of genes would code for generic metabolic
535 processes (*e.g*. Complex I intermediate-associated protein 30 involved in
536 respiratory metabolism, Walker et al., 1992). These functions are essential
537 for cellular stability and adaptation to fluctuating conditions. However, some
538 genes would be involved in more stress-specific responses that might be
539 triggered by exposure to predators (*e.g*. a gene would be involved in the TOR
540 protein complex, well-known to influence response to abiotic stressors;
541 Blackwell et al., 2019; Katewa & Kapahi, 2011). In the same vein, some
542 DEGs were linked to the calcium ion metabolism (*e.g*. by coding for Annexin
543 and Peptidase C2 protein families; Khorchid & Ikura, 2002) and would be
544 involved in the shell synthesis. Calcium being the main component of shell
545 gastropods (Bukowski & Auld, 2014), the modulations of general calcium
546 pathways could influence the expression of anti-predator defences like
547 increase of shell thickness and change in shell shape observed in our study.
548 Our results are consistent with previous studies investigating the functions of
549 DEGs in predator cue environments and showing that genes are associated
550 with defensive structures, metabolism and transcription regulation. Indeed,
551 Hales et al. (2017) and Rozenberg et al. (2015) showed on *D. ambigua* and
552 *D. pulex* that the gene functions mostly explained the observed defensive
553 phenotypic changes (*e.g*. expression of proteins involved in formation of
554 protective structures and in cuticle strengthening) and were also involved in
555 metabolism pathways as for example metabolic proteins required for
556 resource allocation or stress responses. Similar results have been found in
557 larvae of the damselfly *Enallagma cyathigerum* and in the marine copepod

558 *Calanus finmarchicus* where predation risk triggered different gene
559 expression coding for stress proteins and reduced antioxidant defence and
560 for an alteration of lipid metabolism, respectively (Skottene et al., 2020; Slos
561 & Stoks, 2008). Although to relate the functions of DEGs with phenotypic
562 traits is always highly speculative in non-model organisms, our study may
563 highlight some interesting molecular pathways underlying predator-induced
564 WGP in *P. acuta* that would be interesting to study in further detail.

565

566 **Phenotypic changes and gene expression induced by trans-**
567 **generational exposure to predator cues.**

568

569 Our results confirmed the predator-induced TGP. The parental exposure to
570 predator cues significantly influenced the offspring phenotype, in particular
571 morphological traits. F2 snails from exposed parents had thicker and longer
572 shells with narrower apertures than snails from non-exposed parents while
573 they had a similar anti-predator behaviour. These transgenerational effects
574 are consistent with previous studies on this system demonstrating that
575 parental environments shape anti-predator traits of offspring (Beaty et al.,
576 2016; Tariel, Plénet, et al., 2020c). A thicker shell and a larger size increase
577 the sell-crush resistance, and a narrower aperture limits the body snail
578 extirpation by crayfish (Auld & Relyea, 2011; DeWitt, 1998). The longer shell
579 of F2 snails from exposed parents was opposed to the shorter shell of
580 exposed snails in F1. This opposite response is consistent with previous

581 studies (Tariel, Plénet, et al., 2020c) and suggest that snails from exposed
582 parents, by being informed of the risk at a very early stage, may use
583 developmental pathways allowing them to invest in both shell growth to reach
584 a refuge size (Auld & Houser, 2015) and shell thickness to increase crush-
585 resistance (Beaty et al., 2016; Tariel-Adam et al., 2023). The anti-predator
586 behaviour was similar between snails from non-exposed and exposed
587 parents, which is in line with some contrasted results observed in other
588 studies (Tariel, Plénet, et al., 2020a, 2020c). Overall, the expression of anti-
589 predator traits by offspring from exposed parents, even if they are not
590 themselves exposed to predator cues, may allow them to anticipate future
591 predation risk and to increase their survival (MacLeod et al., 2022; Tariel,
592 Plénet, et al., 2020b).

593

594 At the molecular level, the predator-induced TGP was associated with 23
595 DEGs (17 up-regulated and 6 down-regulated) according to the parental
596 environments. The low number of DEGs in F2 generation contrasts with other
597 studies on various factors (Clark et al., 2019; Ledón-Rettig, 2023) and on the
598 parental exposure to predator cues (Hales et al., 2017; Mommer & Bell,
599 2014; Stein et al., 2018). For example, Stein et al., (2018) found 322 DEGs
600 in threespined stickleback *G. aculeatus* offspring when predation risk was
601 experienced by the father. In *D. ambigua*, individuals from predator-exposed
602 parents and grand-parents had 233 and 170 DEGs respectively compared
603 to individuals from non-exposed parents (Hales et al., 2017). Although this
604 contrast may result from a less stringent method of DEGs selection (no LFC

605 filtering, *e.g*. Stein et al. 2018), it may also question whether our experiment
606 has captured the entire transgenerational transcriptomic response.

607 Firstly, gene expression is investigated in only one tissue (i.e. the mantle skirt
608 that synthesizes the shell) in this study while in many studies it was studied
609 in whole organs (*e.g*. Stein et al. 2018) or whole individuals (*e.g*. Hales et al.
610 2017). This may drastically decrease the number of DEGs detected,
611 capturing only a specific part of gene expression changes caused by parental
612 environments. However, this tissue-specific approach is particularly relevant
613 in systems like ours as it allows to specifically study gene expression in the
614 tissue at the origin of phenotypic traits (i.e. shell modifications in this study).

615 Secondly, this study only investigated the gene expression in offspring that
616 were not themselves exposed to predator cues, which did not allow to gain
617 information whether the gene expression associated with parental
618 environments is modulated by developmental one. In *P. acuta*, there is
619 evidence that parental and developmental environments interact to shape
620 the offspring phenotype: the resulting phenotypic patterns showed much
621 stronger TGP in offspring that had not experienced predator cues themselves
622 (WGP masking TGP for most traits; (Luquet & Tariel, 2016). Although it would
623 be relevant to provide an integrative view of gene expression patterns in all
624 environmental scenarios, our study of DEGs associated with TGP focused
625 on the environmental situation where TGP is the strongest.

626 Thirdly, the lower number of DEGs in F2 could also result from a time lag
627 between changes in gene expression and anti-predator defence induction

628 (Gilbert, 2001). Indeed, the environmental conditions experienced in early
629 life can have important consequences for phenotype later in life (Gilbert,
630 2001; Lee et al., 2013) but also for offspring phenotype (Burton & Metcalfe,
631 2014; Tariel-Adam et al., 2023; Yin et al., 2019). For example, maternal
632 provisioning plays a crucial role in transmitting environmental information to
633 offspring by influencing cytoplasmic components, including mRNAs and
634 proteins, during early embryogenesis (Burton & Metcalfe, 2014). This
635 process can impact gene regulation during the maternal-to-zygotic transition,
636 potentially shaping phenotypic responses before they are detectable in
637 adults. In this study, both differential gene expression and anti-predator
638 defences in offspring have been investigated at the adult stage while
639 offspring may have integrated the environmental information from their
640 parents early in development engaging them in specific predation risk
641 developmental trajectories. As a consequence, the anti-predator defences
642 observed in offspring from exposed parents may result from gene expression
643 changes early in development that no longer exist at the adult stage, which
644 could also explain why we did not find a clear common core set of genes
645 between WGP and TGP. For example, Mommer & Bell (2014) showed that
646 maternal experience with predation risk influences genome-wide embryonic
647 expression in *G. aculeatus*, an early expression that may have phenotypic
648 consequences later (Sharda et al., 2021). Such a complex interplay between
649 the timing of environmental cue perception, gene expression and phenotypic
650 consequences deserves to be explored in more details, for example by
651 tracking the DEGs throughout the development.

652

653 The functional analysis of DEGs between snails from non-exposed parents
654 and those from exposed parents suggested the same main functions as for
655 WGP, i.e. metabolism and transcription regulation. Genes linked with
656 metabolisms would be mainly involved in response to oxidative stress and
657 carbohydrate metabolism. One gene would be involved in the regulation of
658 cytosolic calcium ion concentration, which might influence the shell synthesis
659 as previously mentioned for WGP. Most genes linked with transcription
660 regulation in F2 would be involved in methyltransferase activity, suggesting
661 that DNA methylations, a well-known bearer of epigenetic information, might
662 be involved in predator-induced TGP (Fallet et al., 2020). Epigenetic
663 mechanisms, such as DNA methylation and histone modifications, are known
664 to contribute to transgenerational inheritance of phenotypic traits (Duncan et
665 al., 2014; Fallet et al., 2020). Recent studies on the freshwater gastropod
666 *Potamopyrgus antipodarum* have demonstrated that differential DNA
667 methylation patterns were associated with heritable morphological
668 differentiations among genetically uniform populations (Smithson et al.,
669 2020; Thorson et al., 2017, 2019). These results suggest that
670 transgenerational inheritance of anti-predator traits in *P. acuta* might be
671 mediated by bearers of epigenetic information and call for more investigation
672 of epigenetic inheritance in the context of predator-induced plasticity.

673

674 **Comparison of differentially expressed genes associated with WGP**
675 **and TGP**

676 The number of DEGs associated with TGP was lower than those for WGP.
677 As mentioned above, this may occur because we only investigated DEGs at
678 the adult stage or because F2 snails did not experiment direct exposure to
679 predator cues. Surprisingly, the DEGs associated with WGP and TGP were
680 not overlapped, suggesting that predator-induced TGP and WGP do not
681 share common genes. This is consistent with Hales et al. (2017) showing
682 that the DEGs for predator-induced WGP and TGP in *D. ambigua* were
683 largely distinct. On the contrary, Stein et al. (2018) also showed that there
684 were unique sets of genes to the different forms of plasticity but also found a
685 common core set of genes in *G. aculeatus*. Despite the absence of common
686 genes in our study, the DEGs in F2 seem involved in the same main functions
687 that those in F1, i.e. metabolism and transcriptional regulation.
688 Consequently, these results could suggest that common functions are used
689 for predator-induced WGP and TGP via different genomic pathways. This
690 would have important implications on the evolution of predator-induced
691 plasticity as it suggests that WGP and TGP can evolve independently.

692

693 **Conclusion**

694 This study confirmed the predator-induced WGP and TGP in *P. acuta*,
695 showing that anti-predator traits (escape behaviour, shell thickness, shell
696 length and shell shape) are induced when snails were exposed during their

own development or when they originated from parents exposed to predator cues (but not exposed themselves). These within- and transgenerational phenotypic responses were both associated with differential patterns of gene expression. However, the transcriptomic signal was lower for TGP (a lower number of DEGs) and surprisingly no common genes were shared between WGP and TGP although the main functions would be similar (metabolism and transcription regulation). Expression analysis showed that only a small set of genes (112 in F1 and 23 in F2) had a great response with high differential expression levels in both generations. Different hypotheses have been proposed to explain the different transcriptomic patterns observed between predator-induced WGP and TGP and called for further investigations allowing to address the limitations of this study in order to track the transcriptomic signal throughout the development, across different tissues and environmental scenarios. This will provide a more integrative view of underlying molecular mechanisms of WGP and TGP addressing an important gap of knowledge on important processes for adaptation to changing environments.

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721 and E.L. performed the experiments. L.K. performed RNA extractions and
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723 performed bioinformatic molecular analyses. L.D. wrote the first version of
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734 https://www.ebi.ac.uk/ena/browser/view/PRJEB87216]. Additionally, scripts
735 used for the analysis, along with the non-genomic data, have been made

available on Zenodo. The repository can be accessed at [10.5281/zenodo.15043538].

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