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 still misunderstood. 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 shown similar responses. Within- and transgenerational plasticity were accompanied by the differential expression of 78 genes (77 up- and 1 down-regulated) and 14 differentially expressed genes (13 up- and 1 down-regulated), respectively. Within- and transgenerational plasticity did not share common differentially expressed genes but some differentially expressed genes in F1 are also highly expressed in F2 and overall, the molecular functions were similar, involving metabolism and transcription regulation. The quantitatively low molecular response associated with transgenerational plasticity suggests that gene expression changes induced by parental exposure may have occurred early in development and have decreased at adult stage.

52 **INTRODUCTION**

53

54 Phenotypic plasticity is the ability of a genotype to produce alternative phenotypes under different
55 environmental conditions (Pigliucci, 2005). Plastic responses are widespread across the phylogenetic
56 spectrum (e.g. in plants: Galloway & Etterson, 2007; in amphibians: Luquet et al., 2015; in fishes: Segers
57 & Taborsky, 2011; in ostracods: Walsh et al., 2016), and are a major concern in evolutionary biology as
58 they can be adaptive, i.e. increasing fitness of organisms facing environmental changes (Pigliucci, 2005).
59 Phenotypic plasticity classically refers to within-generational plasticity (WGP), meaning that it occurs
60 within one generation. Recently, plasticity has also been found to occur across generations
61 (transgenerational plasticity, TGP) where the phenotype of an organism is influenced by the
62 environment experienced by the previous generations (Bell & Hellmann, 2019). Interest in TGP has
63 grown rapidly in recent years with evidence that TGP is at least as widespread as WGP (Jablonka & Raz,
64 2009; Tariel, Plénet, et al., 2020b). Although there are numerous examples of TGP generating
65 seemingly adaptive offspring phenotypes, its adaptive role is expected to be more challenging than
66 that of WGP due to patterns of environmental variation across generations (Colicchio & Herman, 2020).
67 Indeed, it requires a strong temporal autocorrelation between environments of distinct generations to
68 be selected (Ezard et al., 2014; Kuijper & Hoyle, 2015; Prizak et al., 2014) and a transmission system
69 that allows the induction of adaptive phenotypes across generations (Fallet et al., 2020). The
70 inheritance mechanisms appear diverse, from epigenetic changes to parental transmission of
71 cytoplasmic elements (small RNAs, nutrients, hormones, proteins), parental care and cultural
72 transmission (Bell & Hellmann, 2019; Danchin et al., 2011).

73 Regardless of the transmission mechanisms, they should lead in the offspring to the modification of
74 the expression of some of the genes involved in the phenotypes, at least in some tissues and at some
75 stages during the development. While transgenerational plasticity studies encompass all aspects of
76 phenotype (including physiological states, morphological traits, behaviour and life-history traits),

transgenerational plastic response at the molecular level is certainly the least studied. This is yet a necessary first step to unravel the molecular mechanisms of plasticity in general and their transgenerational inheritance. The most compelling explorations of changes in gene expression profiles in response to both developmental (WGP) and parental (TGP) environments come from the studies of Hales et al. (2017) on *Daphnia ambigua* and Stein et al. (2018) on three spined stickleback (*Gasterosteus aculeatus*). These studies compared gene expression between individuals that had been exposed to predator cues relative to unexposed individuals within a generation (WGP genes) and among their offspring (descendants of unexposed or exposed parents; TGP genes). Stein et al. (2018) found that the same group of genes changed their expression in offspring whether the predation risk was experienced by the offspring (TGP), the father (WGP), or both. This suggests that a core set of genes is consistently affected by predation risk regardless of who experiences it. On the other hand, Hales et al. (2017) found that changes in the expression of genes that respond to TGP differed depending on whether exposure to predation was experienced by parents or grandparents. They also showed that very few genes were common to both WGP and TGP. Note that Clark et al. (2019) also showed that TGP gene expression profiles of larvae of sea urchin (*Psammechinus miliaris*) spawned at low pH from pre-acclimated adults differed to those of larvae produced from adults exposed to ambient pH, but the authors did not explore the WGP genes. These few results highlight the necessity to investigate together WGP and TGP at the molecular level. Beyond the inheritance mechanisms allowing organisms to memorise past environmental conditions to adjust their future phenotype, we need to know whether the two forms of plasticity are distinct at the molecular level and potentially free to evolve independently of one another (Bell & Stein, 2017).

Predation risk is a major selection pressure for most species and results in the evolution of defences in prey (Kikuchi et al., 2023). As predation risk often varies over time and space, it favours the evolution of phenotypic plasticity in defences (Reger et al., 2018; Tariel, Luquet, et al., 2020; Viney & Diaz, 2012). Indeed, while some defences against predators are constitutive, plastic defences are only induced when prey detect some predator cues in their environment (e.g. visual cues, chemical cues such as predator

odours, or alarm signals released by conspecifics). These induced defences are classic examples of adaptive WGP: prey can increase their fitness by producing defences only when predators are present. The links between prey-predator interactions and WGP have been studied for a long time (e.g Tariel et al., 2020; Tollrian, 1995). Plastic defences can also be induced in offspring after their parents or more distant ancestors have detected predator cues. This transgenerational induction of defences was first highlighted in Daphnia morphology by Agrawal et al., (1999) and has since been repeatedly shown in multiple species and defences (review in MacLeod et al., 2022; Tariel et al., 2020).

In this context, this work aims to unravel the molecular mechanisms underlying the expression of WGP and TGP of defences in the context of predator-prey interactions. We used *Physa acuta*, a freshwater snail known for their within- and transgenerational anti-predator defences such as escape behaviour (snails crawl out of the water when they or their parents detect predator cues; Luquet & Tariel, 2016; Tariel et al., 2020a; Turner et al., 1999), change in shell size and shape, increase in shell thickness and overall increase in shell-crush resistance (*e.g.* shell harder to handle or to crush by predators;(Auld & Relyea, 2011; Beaty et al., 2016; DeWitt, 1998; Tariel et al., 2020c). We combined a two-generation experiment to generate WGP and TGP of defences (behavioural and morphological responses as previously done in e.g. Luquet & Tariel, 2016) with a transcriptomic investigation. We used transcriptome sequencing and assembly to specifically investigate the transcriptome mantle skirt gene expression (mantle skirt is the tissue that synthesises the shell). We expected that gene expression should be affected by current (WGP) and parental (TGP) exposure to predator cues. More specifically, as WGP and TGP induce similar phenotypic responses (e.g. increase in shell thickness, (Tariel et al., 2020), we expected the two processes share the same core set of genes.

125 **MATERIALS & METHODS**

126

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

128 Adult *P. acuta* snails were collected from a wild population in a lentic backwater of the Rhône River
129 (Lyon, France, 45° 48'6"N, 4° 55'33"E) in September 2018. Individuals from this population have been
130 repeatedly used in previous studies on predator-induced plasticity (Luquet & Tariel, 2016) and are
131 naturally exposed to predation by the crayfish *Faxonius limosus*. The experiment was conducted in our
132 laboratory in a temperature-controlled chamber at 25 °C with a photoperiod of 12/12h. The wild snails
133 constituting the F0 generation were pooled in a 10 L plastic box filled with dechlorinated tap water
134 (control water hereafter) and interbreed overnight. They were then isolated in 80 mL plastic boxes (4.5
135 x 6 cm) filled with control (without predator cues) water to lay eggs for 3 days. Eggs within boxes
136 constitute F1 matrilines. They developed until hatching at 25°C (ca. seven days). Siblings of each F1
137 matriline developed for 10 days in control water. Then, 10 siblings of 20 F1 matrilines were split into
138 two developmental environments: five siblings remained in control (F1C) water and five siblings moved
139 to predator-cue (F1P) water. F1 siblings developed together in 80 mL plastic boxes for seven days before
140 being isolated in the same environment (control or predator-cue water) and kind of plastic boxes for
141 24 days (i.e. 41 days old snails from hatching to measurements). At the end, one F1 snail per matriline
142 and treatment was frozen at -80°C for molecular analysis and all remaining F1 snails were measured
143 (see § Trait measurements).

144 To generate the F2 generation, we pooled F1 snails from the same treatment in an aquarium filled with
145 control water and let them copulate for 24h. As previously, F1 snails were then isolated and laid eggs
146 for 3 days to produce the F2 matrilines. F2 snails from the two parental environments (C and P) were
147 all reared in control water (noted F2CC and F2PC thereafter) with the same protocol steps as previously
148 for F1 snails, except that we measured F2 snails at an older age than the F1 generation because of a

149 slower growth. As for F1, one F2 snail per matriline and treatment was frozen for molecular analysis
150 and the others were measured.

151 Snails have been fed *ad libitum* with boiled and mixed lettuce. Water (according to treatment) and food
152 was renewed twice a week. The predator-cue water was water in which *F. limosus* crayfish have been
153 raised (one crayfish/5L), fed with snails, for 3 days and in which we smashed *P. acuta* adult snails (one
154 snail/5L) for 1h before using the predator-cue water (these smashed snails were then used as crayfish
155 food).

156

157 **Behavioural and morphological responses**

158 In order to check that predator cue exposition triggered WGP and TGP, we measured escape behaviour
159 and shell morphology on F1 and F2 snails (339 snails: F1C=76, F1P=73, F2CC=89, F2PC=101). Crawling
160 out of the water (position above the water surface) is considered as allowing snails to escape from
161 benthic predators such as crayfishes (DeWitt, 1998; Tariel et al., 2020a). Shorter and narrower shell and
162 aperture dimensions and thicker shell are adaptive anti-predator responses (Auld & Relyea, 2011).
163 These phenotypic traits have been recurrently involved in transgenerational responses of *P. acuta* to
164 predator cues (Beaty et al., 2016; Luquet & Tariel, 2016).

165 *Escape behaviour* - The position above on or below the water surface of each individual was recorded
166 in their rearing vials one week before measurements for each generation, four hours after water
167 change.

168 *Shell thickness, shell length and morphology* - Shell thickness was directly measured with an electronic
169 calliper at the nearest 0.01 mm at the edge of the aperture. After, a photograph of each snail (aperture
170 upwards) was taken with an Olympus SC50 camera installed on an Olympus SZX9 binocular and its
171 Olympus DF PLAPO 1X-2 objective at a x8 magnification. Snail shell length, shell width, aperture length
172 and aperture width were measured using ImageJ (Schneider et al., 2012). To estimate potential changes

in global snail shell morphology according to treatment, we used geometric methods (Gustafson et al., 2014). 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, resulting in 336 snails (F1C=76, F1P=72, F2CC=89, F2PC=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.

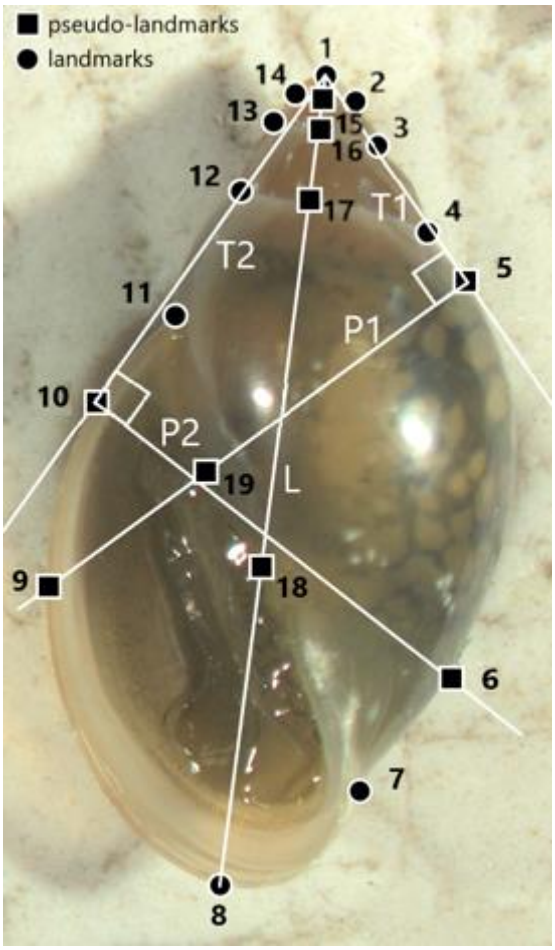


Figure 1: Locations of the different landmarks (circle symbol) and pseudo-landmarks (positioned using a geometric method, square symbol) on *P. acuta* shell. Pseudo-landmarks have been created using a geometric method: first, two tangents (T1 and T2) have been drawn from landmark 1 (apex). T1 touches 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

Escape behaviour - The effects of predator cues on escape behaviour (i.e. snail position above/on or below the water surface) were analysed using generalised linear mixed models (GLMM) assuming a binomial distribution (logit link function). To test for WGP and TGP in F1 and F2 snails respectively,

200 treatment (parental treatment) was a fixed effect; family was considered as a random intercept. We
201 tested significance of fixed and random effects with likelihood ratio tests.

202 *Shell thickness, shell length* - To analyse the effects of predator cues on shell length and shell thickness,
203 we performed linear mixed models with treatment (predation; parental treatment for F2) as fixed effect
204 and family as a random intercept. We used restricted maximum likelihood estimation and Kenward and
205 Roger's approximation for degrees of freedom. We tested significance of fixed effects with type II F-
206 tests (Kuznetsova et al., 2017) and significance of random effect with likelihood ratio test.

207 Linear mixed models were performed using the lme4 package (Bates et al., 2014) on R software (version
208 R-4.4.1 for Windows, RStudio Team, 2020).

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

213

214 **Molecular biology analysis**

215 We dissected 30 snails (F1C = 5, F1P = 5, F2CC = 10, F2PC = 10) to extract the mantel skirt, the tissue
216 that synthesises the shell. Total RNA of the mantle was extracted by adding TRI Reagent (Molecular
217 Research Center MRC, TR118) to the samples and by homogenising tissues on ice with pistons and
218 agitator (Motor Mixer Clearline ref.045011 Dutscher). All remaining steps were carried out according
219 to the manufacturer's protocols (Molecular Research Center MRC, TR118). Then, the RNA was treated
220 with Turbo DNase enzyme (Turbo DNA free kit, Invitrogen, AM1907) and assayed by fluorescence using
221 a Qubit fluorometer (Qubit® RNA HS Assay Kits Molecular Probes, Invitrogen, Q32855). Library
222 construction was carried out using the NEBNext Ultra II RNA Library Prep Kit for Illumina (Biolabs New
223 England, NEB E7770, E7490, E7600). Libraries were randomly splitted in three groups and sequenced

224 on three lanes with an Illumina® HiSeq4000™ machine on the GenomEast platform hosted at the
225 IGBMC (GenomEast Platform – Institut de Génétique et de la Biologie Moléculaire et Cellulaire IGBMC,
226 Illkirch, France), resulting in 50 bp long single-end reads.

227

228 **Differential gene expression analysis**

229 FASTQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/)) was used to assess the quality
230 of the reads. We used a reference genome ORF *P. acuta* available on NCBI (Isolate: Inbredline_101_S28.
231 RefSeq: GCF_028476545.12). Gene expression levels were assessed on annotated transcripts using
232 Kallisto (-l 50 -s 10 options; (Bray et al., 2016). We used rounded effective counts as input for
233 differential gene expression using the DESeq2 package (Love et al., 2014), which performs differential
234 gene expression analysis using negative binomial distribution and shrinkage estimation for dispersions,
235 and outputs expression fold changes to improve the interpretability of estimates. We specified control
236 or predation treatment in the model (design formula: "design = ~treatment") within each generation
237 independently. Firstly, we checked for any structures in the dataset (normalised count table) by
238 performing a principal component analysis (PCA; DESeq2) on the 500 most differentially expressed
239 genes) across samples. Secondly, we used the Wald test (DESeq2 package) to test the significance of
240 differentially expressed genes between the control and predation treatment; we have considered a
241 significant differential expression when adjusted p-value < 0.05 and absolute Log2FoldChange (|LFC|)
242 greater than 3 (LFC is the logarithm base 2 of the fold change in gene expression between two
243 experimental conditions, providing a measure of both the magnitude and direction of differential
244 expression). In addition, we have compared the observed numbers of genes differentially expressed in
245 F1 and F2 to an expected distribution (generated by 1000 permutations) under the null hypothesis (i.e.
246 the differential expressions observed are due to the stochasticity of gene expression between
247 individuals). Thirdly, we determined the potential gene functions and the associated protein families
248 of all significant differentially expressed genes using the InterPro EMBL-EMI database (Paysan-Lafosse

249 et al., 2023). Finally, we investigated whether the differentially expressed genes (|LFC| > 3) in F1
250 parental generation were also differentially expressed in F2 offspring generation by comparing
251 heatmaps. We also compared the mean LFC of differentially expressed genes in F1 versus all the genes
252 expressed in F2 using a Wilcoxon test.

253

254 **RESULTS**

255

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

257 *Within-generational Plasticity* - In F1 parental generation, the proportion of crawling out increased
258 significantly in predator-cue treatment from 7.9 % of snails out of the water in control treatment to
259 93.15 % in predator-cues ($F[1,19.43] = 321.82$, $p < 0.001$; Fig. 2A). Shell length significantly decreased
260 (-6.3%) in predator-cue treatment in F1 generation ($F[1,128.71] = 15.46$, $p < 0.001$; Fig. 2B). Shell
261 thickness significantly increased in predator-cue treatment (+18.8%; $F[1,129.58] = 4.02$, $p = 0.047$; Fig.
262 2C). The family random effect was always non-significant (behaviour: $\chi^2[1] = 0.690$, $p = 0.406$; shell
263 length: $\chi^2[1] = 19.022$, $p<0.001$; shell thickness:$\chi^2[1] = 2.6116$, $p = 0.106$).

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

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, exposed shells 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, non-exposed shells have a wider aperture at the base of the external edge and smaller spires.

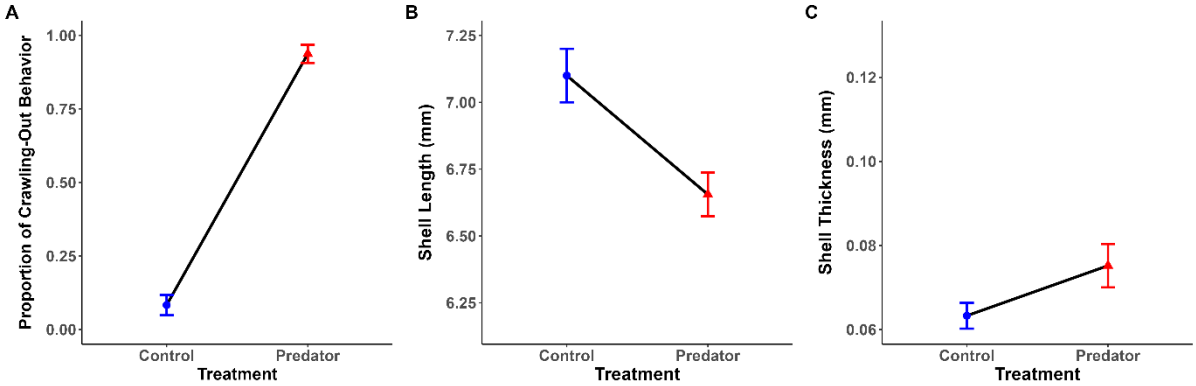


Figure 2: Effect of current exposure to predator cues (WGP) on crawling-out behaviour, shell length and shell thickness in the F1 parental generation of *P. acuta*. Non-exposed and exposed to predator-cue treatments are symbolised with blue circles and red triangles, respectively.

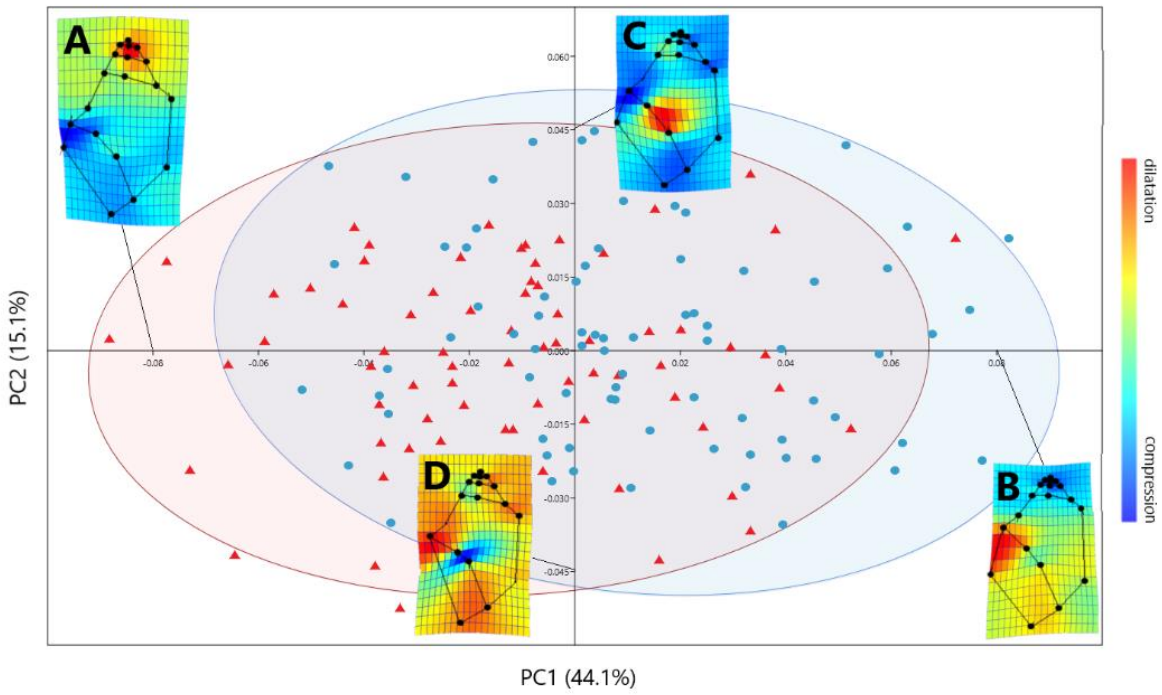


Figure 3: Shape variation of *P. acuta* snails from generation F1C (blue circle symbol) and F1P (red triangle symbol) to predator cues. Panels A, B ,C, D represent deformation grids modelizing shell shape on the extreme ends of the PCA axes. The 95% confidence ellipses are represented for non-exposed and exposed to predator-cue treatments.

Transgenerational plasticity - In F2 offspring generation, the proportion of crawling-out marginally increased in snails from predator-exposed parents, from 3.3% of snails out of the water from parental non-exposed treatment to 8.75% from predator-exposed parents ($F[1,20.31] = 3.929$, $p = 0.061$; Fig. 4A). Snails from predator-exposed parents were significantly longer (+4.9%) and had a thicker shell (+16.9%) than those from non-exposed parents ($F[1,170.87] = 7.55$, $p = 0.006$ and $F[1,174.11] = 9.858$, $p = 0.002$, respectively; Fig. 4B-C). The family random effect was always non-significant (behaviour: $\chi^2[1] = 0.022$, $p = 0.882$; shell length: $\chi^2[1] = 31.685$, $p < 0.001$; shell thickness: $\chi^2[1] = 1.882$, $p = 0.17$).

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. 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.

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 both parental environments (PC2: $t = 0.59$, $p = 0.55$).

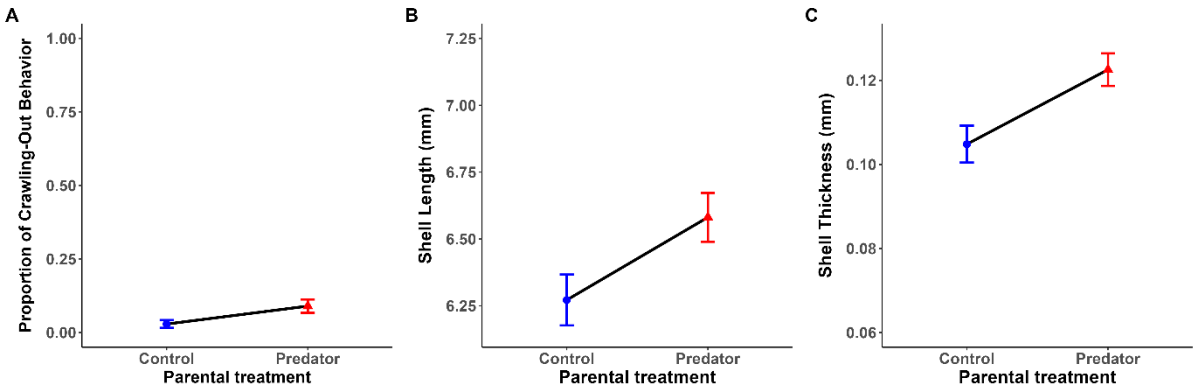


Figure 4: Effect of parental exposure (TGP) on crawling-out behaviour, shell length and shell thickness in the F2 offspring generation of *P. acuta*. Non-exposed and exposed to predator-cue treatments are symbolised with blue circles and red triangles, respectively.

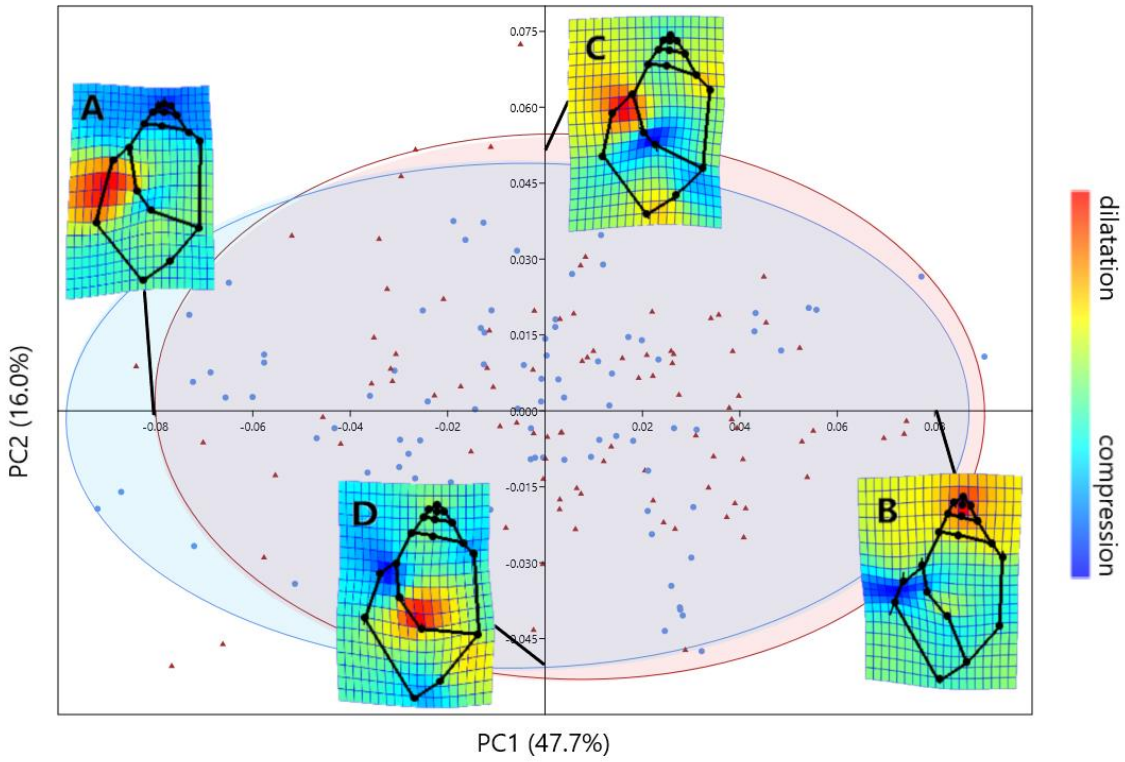


Figure 5: Shape variation of *P. acuta* snails from generation F2C (blue circle symbol) and F2P (red triangle symbol) to predator cues. Panels A, B ,C, D represent deformation grids modelizing shell shape on the extreme ends of the PCA axes. The 95% confidence ellipses are represented for non-exposed and exposed to predator-cue treatments.

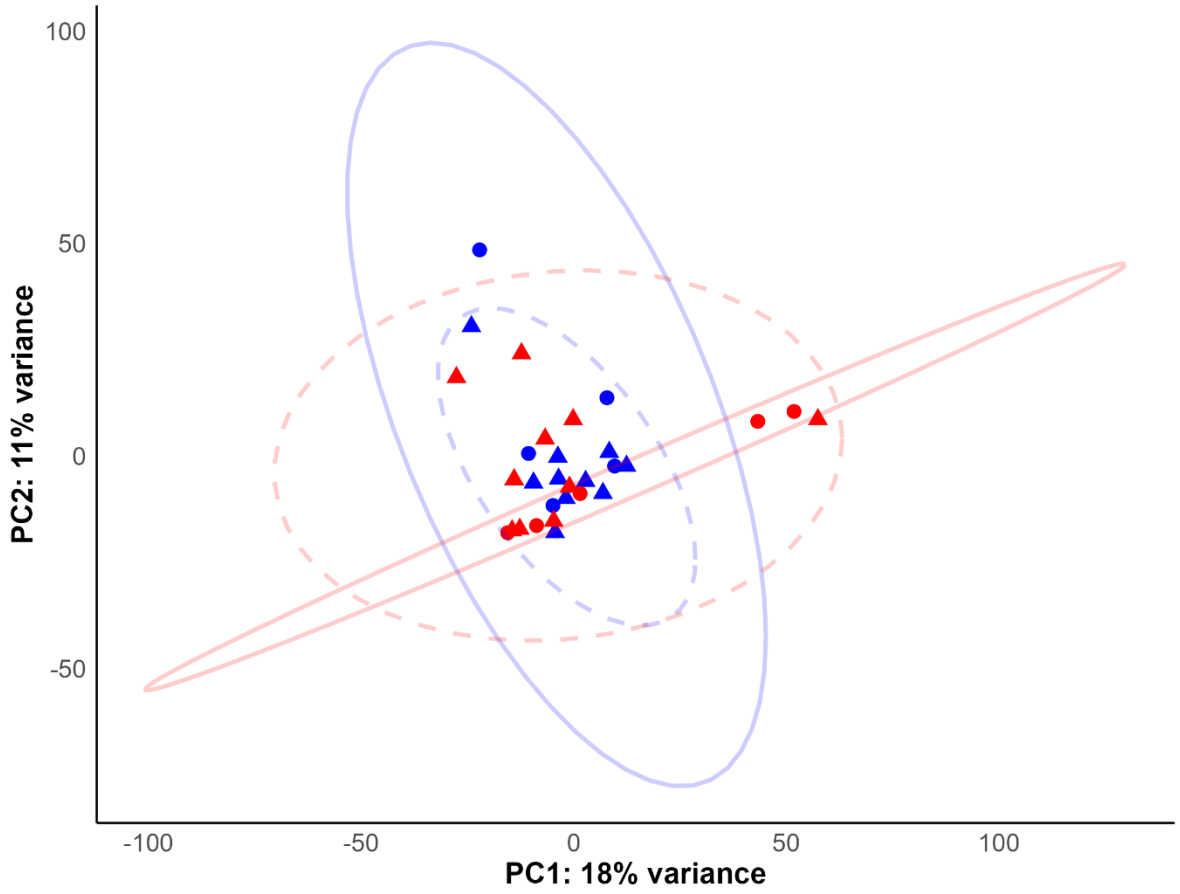
318 **Differential gene expression induced by predator-cues**

319 A principal component analysis (PCA) was performed to visualise the grouping of read counts and to
320 help identify patterns on the first 500 most differentially expressed genes. The first and second principal
321 components (PC1) explained 18% and 11% of the variance respectively (Fig. 6). The plot did not indicate
322 an evident clustering between the control and predator-cue groups or between the F1 parental and F2
323 offspring generations (Fig. 5).

324 The differential expression analysis considering the effect of predator-cue exposure in the F1 parental
325 generation revealed a total of 78 genes with a differential expression (77 up-regulated genes and 1
326 down-regulated gene; Fig. S1). At the F2 offspring generation, the differential expression analysis
327 considering the parental effect of predator-cue exposure showed a total of 14 genes with a differential
328 expression (13 up-regulated and 1 down-regulated gene; Fig. S1). The permutation analyses confirmed
329 that the observed number of up-regulated genes in F1 and F2 are higher than under the null hypothesis
330 (Fig. S2).

331 The differentially expressed genes in F1 and in F2 were mainly involved in metabolism processes (e.g.
332 carbohydrate metabolism, calcium metabolism, fatty acid metabolism) and transcriptional regulation
333 (Fig. 7; see Tables S1 and S2 for details).

334 The comparison of the differentially expressed genes in F1 and in F2 revealed that the 77 up-regulated
335 genes in F1 were not differentially expressed in F2 offspring generation (Fig. 8, the differentially
336 expressed genes in F2 were not expressed in F1 see Fig. S3, an overall comparison of LFC F1 against LFC
337 F2 is shown in Fig. S4). However, we showed that the mean LFC of the 77 up-regulated genes in F1 was
338 significantly higher than the mean LFC of expressed genes in F2 (mean LFC = 0.172 vs 0.023; W =
339 748633, $p < 0.001$). Among the 77 up-regulated genes in F1, 7 were not expressed in F2, 64 were not
340 differentially expressed between non-exposed and exposed parental treatments with a very low LFC
341 (LFC = 0.05) and 6 were not differentially expressed (adjust p-value > 0.35) but with a high LFC (LFC
342 from 1.13 to 3.47; see Table S1).



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344 **Figure 6:** PCA plot of the 30 transcriptome samples of *P. acuta* snails showing the divergence in differential expression

345 among the first 500 most DE genes. Non-exposed and exposed to predator cue individuals are represented in blue and red,

346 respectively. F1 individuals are represented by circles and plain ellipses and F2 individuals by triangles and dotted ellipses.

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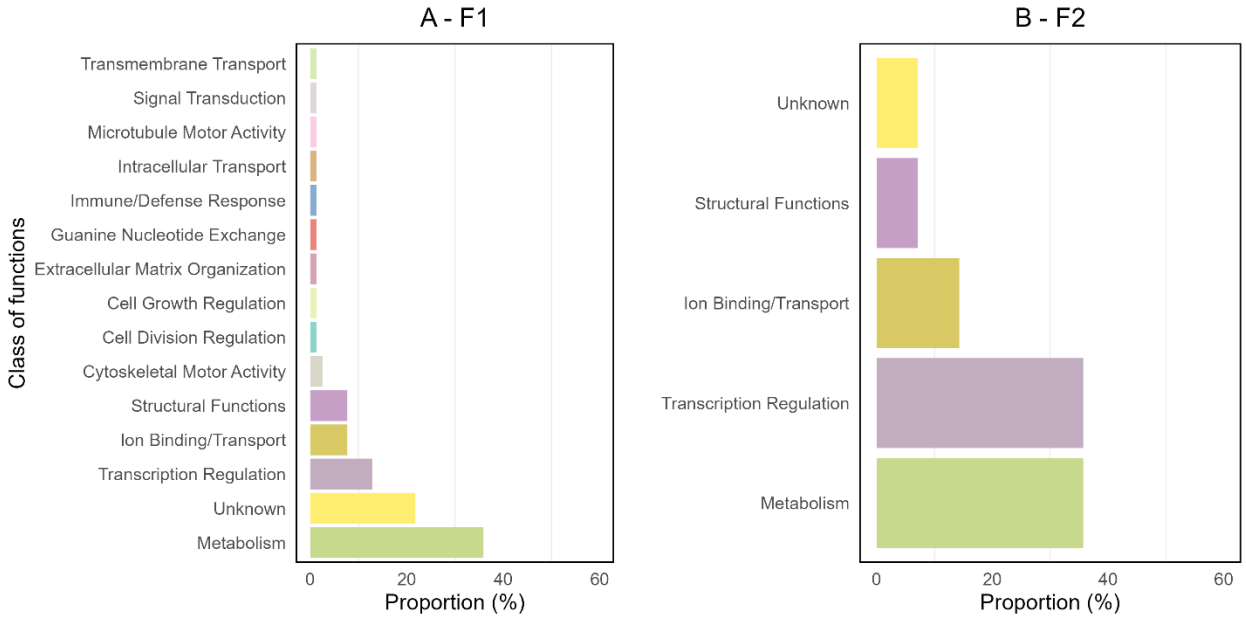

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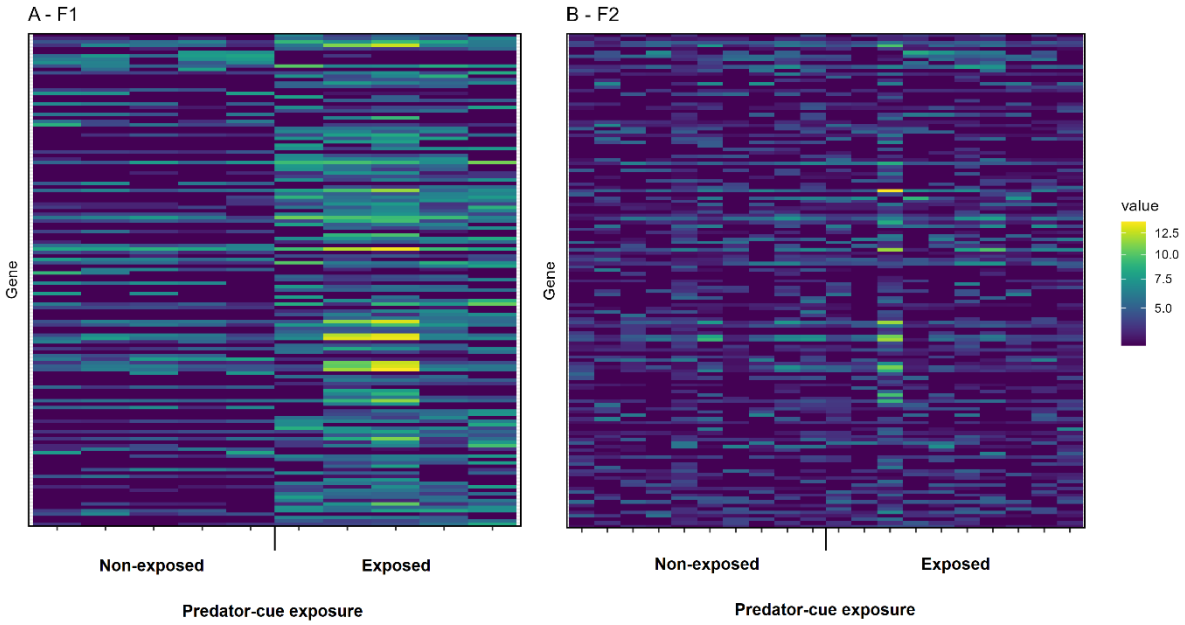
350 **Figure 7:** Proportions of classes of functions of the differentially expressed genes in F1 (A) and in F2 (B) based on the

351 InterPro EMBL-EMI database (Paysan-Lafosse et al. 2022).



352

353 **Figure 8:** Heatmap of read counts of 77 differentially expressed genes (|LFC| > 3) between non-exposed and exposed to

354 predator cues F1 snails (A) and the same genes between F2 snails from non-exposed and exposed parentals (B).

355

356 **DISCUSSION**

357

358 This work aimed to unravel the molecular mechanisms underlying the expression of within- and trans-
359 generational plasticity of anti-predator defences in *P. acuta*. We expected that gene expression should
360 be affected by current (WGP) and parental (TGP) exposure to predator cues, and that both WGP and
361 TGP share the same core set of genes. Exposure to predator cues results in changes in behaviour,
362 growth and morphology confirming that snails perceived predator cues and responded accordingly in
363 F1 (WGP) and that parental exposure influenced the F2 offspring phenotype (TGP). Our study
364 consistently revealed differential gene expression profiles between F1 non-exposed snails and exposed
365 to predator cues (WGP) and also between F2 snails according to parental exposure to predator cues
366 (TGP). However, the number of differentially expressed genes detected for TGP was lower than for WGP
367 and were different.

368

369 **Gene expression induced by within-generational exposure to predator cues**

370

371 As expected, snails exposed to predator cues crawled 85.25% more out of the water, had shells 18.8%
372 thicker, were 6.3% smaller and developed slender shells with longer spires and narrower aperture
373 width than unexposed snails. These behavioural and morphological responses are supposed to be
374 adaptive as they are consistent with an escape behaviour and an increase in shell crush resistance
375 (Tariel et al., 2020c; Tariel-Adam et al., 2023), increasing snail survival in face of crayfish predation
376 (Auld & Relyea, 2011). These predator-induced changes were associated with distinct patterns of gene
377 expression with 78 differentially regulated genes (77 up-regulated and 1 down-regulated genes).
378 Overall, this is consistent with several studies that explored the changes in gene expression induced by
379 the environment (e.g. Aubin-Horth & Renn, 2009; Herman & Sultan, 2011). In the context of predator-

380 prey interaction, the main knowledge about the transcriptomic responses of preys to predators comes
381 from studies on Daphnia species. Hales et al., (2017)) in *Daphnia ambigua* and Rozenberg et al. (2015)
382 in *Daphnia pulex* showed that anti-predator defences, including morphological traits, were associated
383 with 48 and 230 differentially regulated genes respectively. In *Daphnia galeata*, a species only
384 displaying shifts in life history traits when exposed to predator cues, no differentially expressed genes
385 between environmental groups was revealed (but some differences were detected at the scale of
386 genotypes; Tams et al., 2020, suggesting that traits such as morphological traits could be caused by or
387 correlated to much stronger differential gene expression. In *Daphnia magna*, Orsini et al. (2018)
388 revealed no changes in gene expression associated with a short-term exposure to fish kairomones.
389 Consequently, the number of differentially regulated genes involved in predator-induced responses
390 may depend on the defensive trait type and the strength of predator signals (e.g. cue concentration,
391 duration of exposure).

392 Our results showed that the most differentially up-regulated genes in snails exposed to predator cues
393 were mainly linked to metabolism, including metabolic stress and calcium metabolism. Firstly, 35.9%
394 of the up-regulated genes were coding for generic metabolic processes. For example, Complex I
395 intermediate-associated protein 30 is involved in respiratory metabolism, while a protein from AMP-
396 dependent synthetase/ligase domain is involved in the fatty acid metabolism (DiRusso et al., 2005;
397 Walker et al., 1992). Secondly, four up-regulated genes are involved in metabolic stress as for example
398 the gene coding for a protein belonging to the Regulatory Associated Protein of TOR (Target of
399 Rapamycin) family. This protein complex is well-known for its influence on various metabolic pathways
400 (Loewith et al., 2002; Saxton & Sabatini, 2017; Wu & Storey, 2021) and in particular in response to
401 abiotic stressors such as temperature and oxygen availability (Blackwell et al., 2019; Katewa & Kapahi,
402 2011). Since predation is known to generate metabolic stress in preys (Slos & Stoks, 2008), TOR proteins
403 could represent an important molecular mechanism involved in the anti-predator responses of preys;
404 however, to our knowledge, no studies have yet shown direct relationships between TOR proteins in
405 predator-prey interaction. Consistently with a metabolic stress induced by predator-cue exposure,

406 other metabolic genes (e.g. coding for Sulfhydryl Oxidase ALR/ERV) involved in oxidoreductase activity
407 and oxidative stress response have been identified (Braun et al., 1998). In addition, we found that
408 12,8% of up-regulated genes were linked to the regulation of transcription and gene expression; both
409 molecular processes are known to be intimately associated with metabolic stress (de Nadal et al., 2011;
410 Westfall et al., 2004). For example, a gene was coding for proteins from ARID DNA-binding and Lysine-
411 specific demethylase-like domains, both participating in chromatin remodelling and regulation of gene
412 expression (Kim et al., 2004; Lu et al., 2014). Thirdly, we interestingly found two up-regulated genes
413 coding for Annexin and Peptidase C2 protein families that are playing a role in calcium ion metabolism
414 (Khorchid & Ikura, 2002). Calcium being the main component of shell gastropods (i.e. calcium
415 carbonate in the form of aragonite; Heller & Magaritz, 1983; McKillop & Harrison, 1972), the
416 concentration of calcium in the water is known to influence the shell morphology of *P. acuta* and in
417 particular, the predator-induced plastic response (Bukowski & Auld, 2014). Thus, modulations of
418 general calcium pathways could influence the shell synthesis and be directly linked to anti-predator
419 defences like increase of shell thickness and change in shell morphology observed in this study.
420 Altogether, these results are consistent with previous studies investigating the functions of
421 differentially expressed genes in predator cue environments and showing that genes are associated
422 with defensive structures, metabolism and transcription regulation. Indeed, Hales et al. (2017) and
423 Rozenberg et al. (2015) showed on *D. ambigua* and *D. pulex* that the gene functions mostly explained
424 the observed defensive phenotypic changes (e.g. expression of proteins involved in formation of
425 protective structures and in cuticle strengthening) and were also involved in metabolism pathways as
426 for example metabolic proteins required for resource allocation or stress responses. Similar results
427 have been found in larvae of the damselfly *Enallagma cyathigerum* and in the marine copepod *Calanus*
428 *finmarchicus* where predation risk triggered different gene expression coding for stress proteins and
429 reduced antioxidant defence and for an alteration of lipid metabolism, respectively (Skottene et al.,
430 2020; Slos & Stoks, 2008). Although to relate the differentially gene expression profiles with phenotypic

431 traits is always largely speculative in non-model organisms, our study highlights some interesting
432 molecular pathways to study the underlying mechanisms of predator-induced defences in *P. acuta*.

433 **Gene expression induced by trans-generational exposure to predator cues.**

434 As observed in several previous studies on *P. acuta*, we confirmed the predator-induced
435 transgenerational plasticity as the parental exposure to predator cues significantly influenced
436 some offspring traits but to a lesser extent than WGP (Beaty et al., 2016; Tariel et al., 2020c). Indeed,
437 F2 snails from exposed parents were 5.5% more crawling-out the water and had a 16.9% thicker and
438 4.9% longer shell than snails from non-exposed parents while they did not differ in shell morphology.
439 At the molecular level, we have consistently detected different patterns of gene expression according
440 to the parental environments but based on a low number of differentially expressed genes. Indeed,
441 there were only 13 up-regulated and 1 down-regulated genes. Interestingly, our study showed that the
442 differentially expressed genes in response to parental predator-cue exposure (TGP) were not
443 differentially expressed for WGP in F1, suggesting that predator-induced TGP and WGP do not share
444 common genes. This is consistent with Hales et al. (2017) showing that the genes differentially
445 regulated for predator-induced WGP and TGP in *D. ambigua* were largely distinct. Stein et al. (2018)
446 also showed that there were unique sets of genes to the different forms of plasticity but also found a
447 common core set of genes in *G. aculeatus*. We consistently found that 6 differentially expressed genes
448 in F1 were more expressed in F2 than the average of all other genes in F2, suggesting that some genes
449 important for WGP may remain active in offspring but at a lower level than genes specific to TGP. In
450 particular, one differentially expressed gene in F1 and highly expressed in F2 is involved in the calcium
451 metabolism and potentially in shell construction (transcript XM_059293345.1). We also showed that
452 the differentially expressed genes in F2 are involved in the same functions that those in F1, i.e.
453 metabolism, including calcium metabolism and response to oxidative stress, and transcriptional
454 regulation. Consequently, these results suggest that common functions are used for predator-induced
455 WGP and TGP via both common and different genomic pathways. Thus, we can speculate as Stein et al.

456 (2018) that the shared genes are involved in the similar responses to personal and parental exposure
457 to predator cues (higher escape behaviour and thicker shell) while the specific genes reflect differences
458 in response to cues from different sources (growth difference: shorter shell in parents versus longer
459 shell in offspring). This would have important implications on the evolution of predator-induced
460 plasticity as it suggests that WGP and TGP can evolve independently for some specific responses, while
461 maintaining to some extent a common genetic basis.

462 A small number of genes can have an important impact on the phenotype (e.g. Meltzer, 2005),
463 especially when differentially expressed genes are involved in transcriptional regulation (e.g. Gill, 2003)
464 as observed for TGP in this study (e.g. cytosine-specific C5-methyltransferases, C2H2-type zinc-finger
465 family). However, the low number of differentially expressed genes evidenced for predator-induced
466 TGP may question whether our experiment has captured the entire transgenerational transcriptomic
467 response. Indeed, such a low number of differentially expressed genes in F2 generation strongly
468 contrasts with other studies on various factors (e.g. Clark et al., 2019; Ledón-Rettig, 2023) and on the
469 parental exposure to predator cues (e.g. Brass et al., 2020; Hales et al., 2017; Mommer & Bell, 2014;
470 Stein et al., 2018). For example, Stein et al., (2018) found 322 genes that were differentially regulated
471 in threespined stickleback *G. aculeatus* offspring when predation risk was experienced by the father. In
472 *D. ambigua*, individuals from predator-exposed parents and grand-parents had 233 and 170
473 differentially regulated genes respectively compared to individuals from non-exposed parents (Hales
474 et al., 2017). The quantitatively low molecular response induced by parental exposure to predator cues
475 observed in our study could result from a time lag between changes in gene expression and anti-
476 predator defence induction (Gilbert, 2001). Indeed, the environmental conditions experienced in early
477 life can have important consequences for phenotype later in life (Gilbert, 2001; Lee et al., 2013) but
478 also for offspring phenotype (Burton & Metcalfe, 2014; Tariel-Adam et al., 2023; Yin et al., 2019). In
479 this study, both differential gene expression and anti-predator defences in offspring have been
480 investigated at the adult stage while offspring may have integrated the environmental information from
481 their parents early in development engaging them in specific predation risk developmental trajectories.

482 As a consequence, the anti-predator defences observed in offspring from exposed parents may result
483 from gene expression changes early in development that no longer exist at the adult stage, which could
484 also explain why we did not find a clear common core set of genes between WGP and TGP. For example,
485 Mommer & Bell (2014) showed that maternal experience with predation risk influences genome-wide
486 embryonic expression in *G. aculeatus*, an early expression that may have phenotypic consequences
487 later (e.g. Sharda et al., 2021). Such a complex interplay between the timing of environmental cue
488 perception, gene expression and phenotypic consequences deserves to be explored in more details,
489 for example by tracking the differential gene expression throughout the development.

490

491 **Conclusion**

492 Both WGP and TGP are triggered by exposure to predator cues, involving changes in behaviour and
493 morphology associated with differential patterns of gene expression in F1 and F2 generations of *P.*
494 *acuta*. The differential profiles involved genes implicated in metabolism and transcriptional regulation.
495 In the F2 generation, the low number of differentially expressed genes suggests that either the gene
496 expression change induced by parental environment occurs early in development and decreases at
497 adult stage. Our study evidenced that unravelling the underlying molecular mechanisms of predator-
498 induced TGP requires further investigations and particularly focusing on the early developmental
499 period, a period during which differential gene expression induced by the environment may have a
500 delayed impact on the phenotype.

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Authors Contributions

E.L., S.P. and T.L. conceived the project. J.T., S.P. and E.L. performed the experiments. L.K. performed RNA extractions and libraries. L.D., J.T. and M.H. analysed phenotypic data. L.D., N.S and T.L. performed bioinformatic molecular analyses. L.D. wrote the first version of the manuscript. All authors revised the manuscript.

Competing interests

No competing interests.

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