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Sensitive windows for within- and trans-generational plasticity of anti-predator defences

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Abstract

Transgenerational plasticity could be an important mechanism for adaptation to variable environments in addition to within-generational plasticity. But its potential for adaptation may be restricted to specific developmental windows that are highly sensitive and responsive to environmental cues. Determining these sensitive windows is essential to understand the temporal dynamic of environmental cue detection, phenotype induction and selection. We examined the sensitive windows of both within- and trans-generational plasticity of anti-predator defences in the freshwater snail *Physa acuta*. Parental snails were exposed to olfactory cues of their crayfish predator at different exposure windows: embryonic development, early, mid or late post-embryonic development. Behavioural and morphological defences were then assessed in adult parents and offspring. The sensitive window of within-generational plasticity was the embryonic development, the whole post-embryonic development, or a combination of early-life and late development depending on the defence. This showed that early-life periods of development (embryonic and early post-embryonic) are sensitive windows of within-generational plasticity. However, the sensitive window also persisted until late developmental stages for some defences, providing evidence that the early-life is not the only sensitive window as empirical and theoretical studies often state. There were less sensitive windows for transgenerational plasticity: embryonic and/or mid post-embryonic development. Interestingly, the embryonic period was a sensitive window of transgenerational plasticity for a defence only when it was also a sensitive window of within-generational plasticity for that defence. On the opposite, the mid post-embryonic development was a sensitive window specific to transgenerational plasticity. This suggests that transgenerational plasticity, although linked to within-generational plasticity by the embryonic sensitive window, may also be induced via a specific channel, independent of within-generational plasticity induction and expression. Finally, the late developmental window was never a sensitive window of transgenerational plasticity as it was theoretically expected. This result may be explained by the potential long-term reliability of parental cues in our system. It is worth noting that we did not find any sensitive window for some defences, either because none of them induced the defence or all exposure windows induced the defence in a similar magnitude. Overall, the developmental window of cue exposure shapes within- and trans-generational responses and thus brings complexity to the study of phenotypic plasticity, notably when it comes to determining its adaptive potential.

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Introduction

Within-Generational Plasticity (WGP) occurs when the phenotype of an individual depends on its environment (Bradshaw, 1965; Pigliucci, 2001; Price et al., 2003). By contrast, Trans-Generational Plasticity (TGP) occurs when the phenotype of an individual depends on the environment of its parents or more distant ancestors (Bell and Hellmann, 2019; Galloway and Etterson, 2007; Mousseau and Fox, 1998). Both WGP and TGP may reflect two types of processes, often difficult to distinguish, that are phenotypic responses to environmental cues (information-based plasticity) or carry-over effects to environmental conditions (state-based plasticity) (Bonduriansky and Crean, 2018; Donelan et al., 2020). Information-based plasticity can confer adaptive phenotypes when perceived environmental cues reduce uncertainty about the future selective pressures acting on the induced phenotype and individuals can thus develop the well-adapted phenotype in anticipation (information-based plasticity; Agrawal et al., 1999; Bonamour et al., 2019; Donelan et al., 2020; Jablonka et al., 1995; Leimar and McNamara, 2015). State-based plasticity results from the positive or negative effects of being exposed to favourable or stressful environmental conditions such as resource availability and stressors (Bonduriansky and Crean, 2018; Donelan et al., 2020; Mousseau and Fox, 1998). Both WGP and TGP may depend on when an environment has been experienced during development (Bell and Hellmann, 2019; English and Barreaux, 2020; Fawcett and Frankenhuis, 2015); in other words, the same environment may generate different phenotypic effects depending on whether it was experienced early or late in development. Certain developmental windows are particularly sensitive to environments, i.e. environments during these sensitive periods strongly determine the phenotype (English and Barreaux, 2020; Fawcett and Frankenhuis, 2015).

Many experimental studies have shown that early-life stages (*i.e.* embryonic to early post-embryonic stages far from the adult stage) are the most sensitive windows for the induction of WGP (Burton and Metcalfe, 2014; B Jonsson and N Jonsson, 2014; Snell-Rood et al., 2015) and this result is supported by theoretical models about the adaptive evolution of sensitive windows

(reviewed in Walasek et al., 2022). Sensitive windows in these models depend mainly on how uncertain individuals remain about their true environment during development (in most models the true state of the environment does not change over development; but see Walasek et al., 2022). As individuals usually collect more and more environmental cues during their development, they become confident about the true state of their environment and don't need environmental cues late in life. This should favour sensitive periods early in life rather than later (Judy Ann Stamps and Luttbeg, 2022; Walasek et al., 2022). Other factors than the informational state of individuals could explain the general early-life sensitive window. Several lines of evidence suggest that cell epigenomes are particularly sensitive to environmental conditions at early stages of development (especially embryonic stage) and that such early environmentally-induced epigenetic alterations affect a high proportion of cells in adults (Burton and Metcalfe, 2014; Fallet et al., 2020; Faulk and Dolinoy, 2011; Feil and Fraga, 2012). Finally, development engages individuals in developmental pathways where traits become fixed, meaning that early stages have a greater potential to alter the phenotype than later stages (B Jonsson and N Jonsson, 2014; Snell-Rood et al., 2015). Sensitive windows of WGP in later developmental stages (i.e. close to or at adult stage) have also been observed and could be explained by many factors such as no response time lag, a low benefit of phenotypic specialisation, low costs of phenotype reversal or an increase in cue reliability across development (English and Barreaux, 2020; Fawcett and Frankenhuis, 2015; Groothuis and Taborsky, 2015; Walasek et al., 2022).

We know less about the sensitive windows of TGP compared to WGP. A first sensitive window of TGP could be the late developmental stages in parents due to the reliability of parental cues about offspring environment (Bell and Hellmann, 2019; Donelan et al., 2020; Tariel et al., 2020a). This prediction is supported by theoretical models demonstrating that late-perceived cues are the most reliable about offspring environment because of the short time lag between cue exposure in parents and phenotypic selection in offspring (Leimar and McNamara, 2015; McNamara et al., 2016) but poorly in empirical studies (Yin et al., 2019; Zhang et al., 2020). Sensitive windows of TGP should also be the same as those of WGP, namely the early-life stages. Indeed, windows causing the strongest effects on parental phenotype are the most likely to affect their condition (e.g. body mass, physiology, neural pathways) and their key life-history decisions (e.g. dispersal, habitat choice, age at maturity), and thus are the most likely to cause strong effects on offspring (Donelan et al., 2020; Mikulski and Pijanowska, 2010). In addition, the inheritance of epigenetic states is one likely mechanism of TGP and these modifications are particularly sensitive to the environment in early development (Burton and Metcalfe, 2014; Donelan et al., 2020). This prediction seems supported by the recent meta-analysis of Yin et al. (2019) which has found that the effect of parental environment on offspring phenotype has a stronger effect size when parents were exposed as embryos than exposed as juveniles or adults. As for WGP, determining sensitive TGP windows is relevant to understanding the responses to environments and the temporal dynamics of transgenerational phenotypic induction and selection. However, few experimental studies have explored sensitive TGP windows (but see Deng et al., 2021; Fallet et al., 2020; Radersma et al., 2018; Yin et al., 2019) and only one has studied the sensitive windows of WGP and TGP simultaneously allowing to test the relationship between the two (Mikulski and Pijanowska, 2010).

We have examined the sensitive windows of WGP and TGP in the context of predator-prey interactions. Animals can encounter predators at different periods of their life. To adapt to this variable presence of predators, prey defensive strategy can be based on WGP, the so-called inducible defences (Tollrian and Harvell, 1999). Inducible defences are expressed after prey have detected cues of predator presence in their environment (Tollrian and Harvell, 1999). These inducible defences concern all types of traits: behaviour, morphology, life history, and physiology (Tollrian and Harvell, 1999). Concerning TGP, defences can also be induced in offspring after parents have perceived predator cues and also concern all types of traits (e.g. Agrawal et al., 1999; Keiser and Mondor, 2013; Luquet and Tariel, 2016; Stein et al., 2018). To our knowledge, only four experiments have studied the sensitive windows of WGP on inducible defences using *Hyla versicolor* tadpoles (Rick A. Relyea, 2003), *Helisoma trivolvis* freshwater snails (Hoverman and Rick A. Relyea, 2007), *Daphnia magna* water fleas (Mikulski et al., 2005), *Daphnia longicephala*

water fleas (Weiss et al., 2016); and only one experiment has studied the sensitive windows of TGP of inducible defences using *Daphnia magna* (Mikulski and Pijanowska, 2010).

The aim of our study was to determine the sensitive windows of WGP and TGP using the example of inducible defences in the freshwater snail *Physa acuta*. The WGP of inducible defences of *P. acuta* has been well described. In the presence of crayfish olfactory cues, snails (1) are more often at the surface or out of the water (out of reach of crayfish as crayfish consume snails at the bottom of the water column; Alexander and A. P. Covich, 1991a,b; Alan P. Covich et al., 1994; McCarthy and Dickey, 2002; Turner et al., 1999), and (2) crawl more quickly to the surface (Tariel et al., 2020c). In addition, snails which have perceived crayfish cues during their development (3) have a larger and thicker shell making them more difficult to crush and handle – crayfish feed first on small and thin-shelled *Physa* snails (Alexander and A. P. Covich, 1991b; Josh R. Auld and Rick A. Relyea, 2011; Bukowski and Josh R. Auld, 2014; DeWitt et al., 2000; Rundle and Brönmark, 2001; Salice and Plautz, 2011; Stevison et al., 2016); and (4) reproduce later and at a larger size compared to snails which have not perceived crayfish cues (J. R. Auld and R. A. Relyea, 2008; Josh R. Auld and Rick A. Relyea, 2010; Crowl and Alan P. Covich, 1990). In addition, several studies have shown TGP of inducible defences in *P. acuta*: offspring of parents which have perceived crayfish cues express a higher level of behavioural and morphological defences than offspring of parents which have not perceived crayfish cues (Beaty et al., 2016; Luquet and Tariel, 2016; Tariel et al., 2020c). In our study, a first generation of *P. acuta* snails (F1 parental generation) was reared and exposed to olfactory cues of crayfish presence at different developmental windows: embryonic development, early, middle or late post-embryonic development, or throughout life. When adult, the F1 snails' behavioural and morphological defences were assessed (crawl-out behaviour, snail size, shell thickness and crush resistance) to determine the sensitive windows of WGP. Next, the second generation of snails (F2 offspring generation) from the different parental exposure treatments were reared without crayfish cues. The F2 snails' defences were assessed at the adult stage to determine the sensitive windows of TGP. For WGP, we predict that embryonic and early post-embryonic windows should be particularly sensitive as mainly observed in previous works. For TGP, we proposed two non-exclusive predictions. (1) We predict that the sensitive windows should be the late parental development because theoretical models showed that later perceived cues are the most reliable about offspring future environment. (2) The sensitive window of TGP should be the same as the sensitive windows of WGP, i.e. embryonic and early post-embryonic windows, as strong changes in parental condition and life-history decisions may lead to strong changes on offspring phenotype.

Material and methods

1. Experimental design

1.1. Snail collection and F0 rearing. 256 adult *Physa acuta* snails were collected in a wild population located in a backwater of the Rhône river (Lyon, France, 45.8078° N, 4.99772° E) on the 12th of March 2018. The following part of the experiment took place at our laboratory in a temperature-controlled chamber at 25 °C with a photoperiod of 12/12h. The wild snails laid eggs that formed the F0 generation. From egg to maturity, these F0 snails were only reared in control water (dechlorinated tap water), in groups, and with ad libitum feeding of boiled and mixed lettuce. At maturity, 231 F0 were isolated to lay eggs in small 70-mL individual boxes to insure a box only kept offspring from one F0 individual, i.e. one F1 maternal family per box. 36 h later, the F0 were removed from the boxes and the experimental treatment started on the F1 parental generation.

1.2. Experimental treatment. The F1 parents were randomly assigned to one of the 6 treatments (Figure 1):

- Control: Snails were reared in water without any predator cues during all development.

And 5 treatments with exposure to predator cues at different developmental windows:

- Embryo: Snails were exposed to predator cues for 5 days during embryonic development.
- Early: Snails were exposed for 14 days during early post-embryonic development.

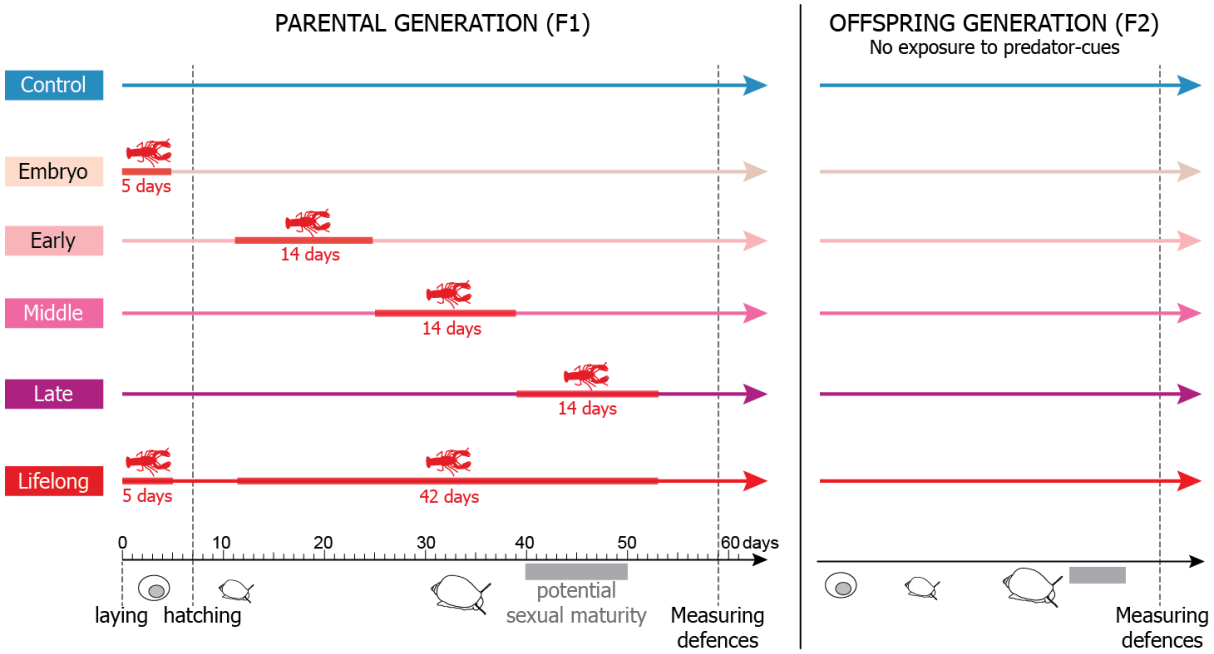

Figure 1 – Experimental treatment and life-cycle of *Physa acuta* at 25°C. The F1 parental generation was split into six treatments with exposure to predator cues at different developmental windows: Control with no exposure to predator cues, Embryo, Early, Middle; Late, and Lifelong. The offspring generation was never exposed to predator cues.

- Middle: Snails were exposed for 14 days during middle post-embryonic development.
- Late: Snails were exposed for 14 days during late post-embryonic development and beyond sexual maturity.
- Lifelong: This treatment combined all windows of exposure; snails were exposed for 5 days during embryonic development and then for 42 days during post-embryonic development.

The choice of exposure duration was based on the life cycle of *Physa acuta* at 25°C to maximise exposure duration during the different developmental stages (Figure 1). The embryonic development is minimum 7 days and Embryo snails were thus exposed 5 days to expose them the maximum amount of time without exposing hatchlings to predator cues. Sexual maturity is a minimum of 1 month after hatching. The exposure was split between 14 days during early post-embryonic development (Early) and 14 days later before sexual maturity (Middle), and finally 14 days for the late post-embryonic development that possibly goes beyond sexual maturity (Late) to stay consistent with the exposure duration of Middle and Early.

The F2 offspring were only reared in water without any predator cues during all their development (Figure 1).

Exposure to predator cues was achieved by rearing snails in water containing predator cues. This predator-cue water was obtained from the rearing water of several *Orconectes limosus* crayfish. Crayfishes are common predators of *P. acuta* snails and the distribution of *O. limosus* crayfish overlaps the natural population from which F0 wild snails come from. These crayfish were individually reared in plastic boxes containing 4 L of water each and fed exclusively with *P. acuta* crushed snails. After collecting the crayfish's rearing water, one crushed snail ($\sim$ 40 mg) per 4L was 'infused' for 1 h. Thus, the predator-cue water contained olfactory cues of crayfish presence, both crayfish kairomones and snail alarm cues.

1.3. Rearing of the F1 parental generation. All F1 snails were reared the same way apart from whether or not they were exposed to predator cues. We randomly assigned 30 F1 maternal families to each treatment. After the F1 maternal families were separated from their F0 mothers, water was changed with predator-cue water for the Embryo and Lifelong treatments, and control water for the other treatments. 5 days later, the water was changed to control water for all

treatments to prevent neonates of the Embryo treatment from being exposed to predator cues (Figure 1). We waited until all F1 snails hatched (6 days later) and started the Early treatment with predator-cue water for the Early and Lifelong treatments, control water for the other treatments. Until the end of rearing, water (control or predator-cue) was changed twice a week for all treatments to refresh predator cues and oxygen. Food was changed at the same time and was made of boiled and mixed salad ad libitum. 14 days later, the Middle treatment started with predator-cue water for the Middle and Lifelong treatments, and control water for the other treatments. F1 snails also began at this time to be reared in larger boxes in which F1 families were mixed (see Supplementary Information 1 for more details on rearing). 14 days later, the Late treatment started with predator-cue water for the Late and Lifelong treatments, and control water for the other treatments. We controlled snail density within aquaria to avoid heterogeneous growth between aquaria (snail density strongly influences growth, which may prevent detecting the effects of the predator treatment). This meant that we had to mix snails from different aquaria within treatments and lose snail identity and aquarium block effect. The Late treatment lasted 14 days and after which the experimental treatment ceased and all F1 were reared in control water for 6 days (i.e. until they were 59 $\pm$ 2 days old). We then randomly chose 40 F1 for each of the six treatments (240 F1 in total). We isolated them in the small individual boxes to keep their identity during defence measurements and to generate the F2 families in separate boxes. F1 laid eggs in their individual boxes during the first 4 days of defence measurements when we measured refuge use (see Supplementary Information 2 for more details on mating and *P. acuta* reproductive system). These eggs constituted our F2 generation with 40 F2 families for each parental treatment.

1.4. Rearing of the F2 offspring generation. F2 were reared in the same way as F1, except that F2 were reared only in control water (Figure 1). They were reared beyond maturity until reaching a sufficient size to measure their defences.

2. Measuring defences

Several defences (Figure 2) were measured on the 240 F1 and 240 F2 adult snails (40 snails x 6 treatments).

- *Refuge use* was scored as a binary variable: 1 if the snail was out of the water or touching the surface, 0 otherwise (Figure 2A). It was measured 4 times in the small individual box of each snail, once in the morning for 4 consecutive days in the F1 generation; and 3 times in the F2 generation. After assessing refuge use, we assessed time to teach the refuge.
- *Time to reach the refuge* was the time taken by the snail to crawl out of the water from the centre of a box, in other words the latency time to reach the refuge zone. For each snail, the time to reach the refuge was measured 4 times in two different test environments: 2 times in control water and then 2 times in predator-cue water. Tests were spaced at least 1 h apart and at most 3 h apart, and took place on the same day. The tests did not take place in the small individual boxes but in specific test arenas which were round plastic boxes of 6.5 cm in diameter and 5.5 cm in height filled with 2 cm of water. At the centre of this arena, a central zone of 2.2 cm diameter was delineated by a line in black marker (Figure 2B). To begin, the snail was placed in the central zone. As soon as the snail left the central zone, we started the timer on JWatcher (Blumstein and Daniel, 2007). The timer was stopped as soon as a part of the snail's body or shell touched the surface to record the time to reach the refuge at the nearest 1 sec. If after 5 min (= 300 sec), the snail had still not touched the surface, the timer was stopped anyway and the test was therefore censored. The snail then returned to its small individual rearing box for some time. Water in the test arena was changed before to measure the next snail. After assessing refuge use and time to reach the refuge, we measured morphological defences.
- *Snail mass*. Total fresh mass (shell + body) was measured with a precision scale at the nearest 0.1 mg.

- *Shell length and width*. Maximum shell length and width were measured with the software ImageJ (Schneider et al. 2012) at the nearest 0.001 mm from a photograph of the shell with its aperture upward taken by a camera mounted on binocular loup.
- *Shell thickness* was measured at the shell aperture edge with a digital calliper at the nearest 0.01 mm.
- *Shell crush resistance*. The crush resistance of empty shells was measured by an automatic device designed for that purpose. The shell was put aperture downward (= based on its aperture) on a force sensor; then a small square piece of metal was moved slowly and steadily downward over the shell by a motor until the shell was completely crushed. The recorded force was initially zero and then picked up until the shell broke. The force recorded at the peak was manually extracted from the force sensor data record at the nearest 0.1 g and represents the force needed to crush the shell.

3. Statistical analysis

Statistical analysis tested whether the developmental window of exposure to predator cues influenced the expression of several defences in adult parents (F1) and adult offspring (F2). Data from the F1 and F2 generations were analysed similarly (see statistical model formulas in Figure 2). Treatment was included in all models as a fixed effect and factorial variable of 6 levels (Control, Embryo, Early, Middle, Late, and Lifelong).

A. *Refuge use* was a binary variable (0 or 1) and was then analysed with a Generalized Linear Model (GLM) using a binomial family with a logit link function. The GLM only includes Treatment as a fixed effect.
B. *Time to reach the refuge* was right-censored at 300 sec because we stopped the test. However it was analysed with a Linear Mixed Model (LMM) rather than a survival model as very few tests were censored: 36 censored tests out of 960 (4 tests per snail x 40 F1 snails x 6 treatments) in F1; 48 out of 960 in F2. The fixed effects of the LMM were Treatment, Test environment (a factorial variable with two levels: control or predator-cue in the water during the test), Repetition (a factorial variable with two levels: 1st or 2nd time a snail encountered a particular test environment), and all interactions. Interactions were not significant at either generations and were therefore removed from the model. The random effect was the snail identity to account for repeated tests on the same snail. To achieve normal distribution required for LMM, time to reach the refuge was log10 transformed. We also multiplied it by -1 as a low time to reach the refuge reflects a high anti-predator behaviour. Parameters of the LMM were estimated with a restricted maximum likelihood procedure. We used the lme4 and lmerTest R packages to implement the LMMs (Bates et al., 2015; Kuznetsova et al., 2017). Time to reach the refuge was repeatable at the parental generation (R = 0.23 (95% CI: 0.19-0.31)) and offspring generation (R = 0.32 (95% CI: 0.28-0.42)). Repeatability R was calculated as follow: $R = \sigma^2_{snail.identity}/(\sigma^2_{snail.identity} + \sigma^2_{residual})$.
C. *Snail size*. Shell length, shell width and the snail mass (cube root transformed) were highly correlated in both F1 and F2 (pairwise Pearson correlation coefficient at least > 0.89). Thus, to reduce the number of variables, a principal component analysis (PCA) was performed using the FactoMineR R package (Lê et al., 2008). The first axis of this PCA summarised well the variability information contained in the three variables (> 95% of the variance; Figure 2C). We interpreted it as an estimation of snail size. The coordinate on the first PCA axis was extracted for all individuals and analysed in a linear model (LM) including only Treatment as a fixed effect. The few missing data were handled with the missMDA R package (Josse and Husson, 2016) but they were not impacting the PCA results.
D. *Shell thickness* was analysed in a linear model (LM) with Treatment as the only fixed effect.
E. *Shell thickness corrected by snail size*. Larger snails are thicker, in other words, there is a correlation between shell thickness and snail size (Pearson correlation coefficient > 0.48). But for snails of the same size, there was still variability in shell thickness; meaning that

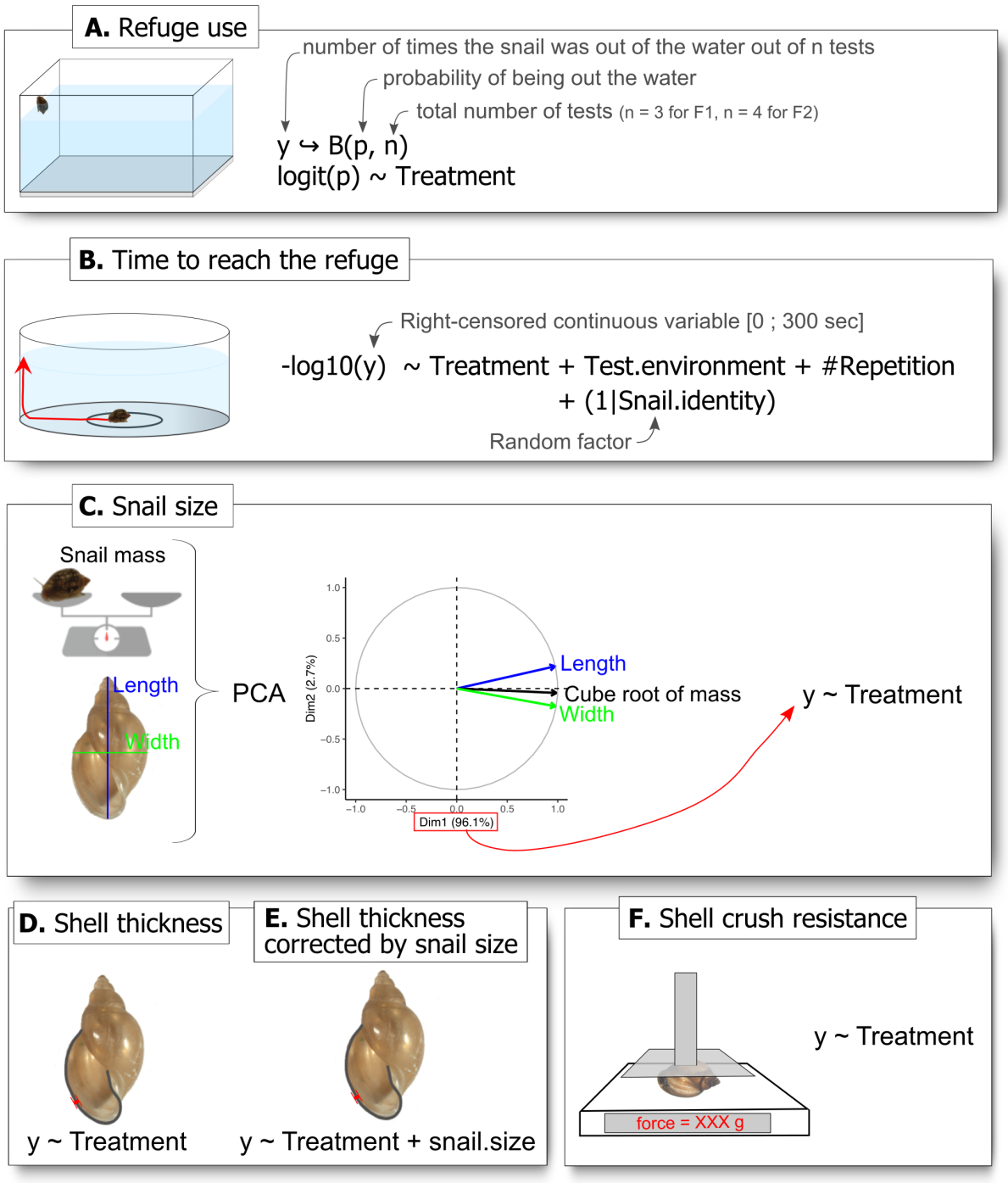

Figure 2 – Inducible defences of *Physa acuta*: measurements and statistical analysis.

a snail can adjust its shell thickness partly independently of its size. To test the effect of treatment on the shell thickness independent of snail size, shell thickness was analysed in another LM including Treatment, snail size, and their interaction as fixed effects. The interaction was not significant at either generation and was therefore removed from the model. To plot shell thickness corrected by snail size, we computed model predicted means using the emmeans R package (Lenth, 2019) in the theoretical case where all snails would be the same size.

F. *Shell crush resistance* was analysed in a linear model with Treatment as the only fixed effect.

To test significance of fixed effects, LMs used type II F-tests, LMMs used type II F-tests with Kenward and Roger's method, and GLMs used a likelihood ratio test (χ^2). To test significance of the random effect, LMMs used a likelihood ratio test (χ^2). For all models, we visually checked normality of residuals and their homoscedasticity across factor levels. We did not remove any outliers. Standardised effect sizes of fixed effects were estimated by the partial η^2 for the LMs and LMMs using the effectsize package (Ben-Shachar et al., 2020); and by the McFadden's Pseudo-R^2 for the GLMs. Both η^2 and R^2 indicate the percentage of variance explained by a fixed effect (for example η^2 = 0.11 for the Treatment fixed effect indicates that 11% of the variation in defence expression is explained by the predator treatment).

When several exposure windows were significantly different from the control treatment, we performed custom post-hoc pairwise contrasts to test whether the defence induction was higher for one/some of these exposure windows compared to the others. The emmeans package was used (Lenth, 2019).

We used R software version 4.0.2 and Rstudio version 2022.2.3.492 (R Development Core Team, 2022; RStudio Team, 2022). We used the ggplot2 R package for graphing (Wickham, 2016), ggpubr to arrange multiple plots (Kassambara, 2020), and the tidyverse suite for dataset manipulation (Wickham et al., 2019).

Results

1. How did the exposure at different developmental windows affect WGP?

Refuge use (Figure 3A; Table 1A) was not significantly influenced by developmental exposure to predator cues.

Time to reach the refuge (Figure 3B; Table 1B) was influenced by the test environment: snails reached the refuge zone on average 19 sec faster when they were tested in an environment with predator cues than without predator cues. Time to reach the refuge was also influenced by developmental exposure to predator cues. Snails exposed to predator cues during their post-embryonic development (Early, Middle, Late, marginal effect for Lifelong) reached the refuge zone 15 sec slower than unexposed snails (Control) whatever the test environment was. Investigating further, there was no significant difference in time to reach the refuge between snails from Early, Middle and Late windows (Table 1B; Figure 5). There was no difference in time to reach the refuge between snails only exposed during their embryonic development (Embryo) and unexposed snails.

Snail size (Figure 3C; Table 1C) was influenced by developmental exposure to predator cues. Snails exposed once during their development (Embryo, Early, Middle, Late) were larger than unexposed snails. Investigating further, snails exposed during their embryonic development (Embryo) were significantly larger than snails exposed during their post-embryonic development (Early, Middle, Late), but there was no significant difference in size between snails from Early, Middle and Late windows (Table 1C; Figure 5). The shell of Embryo snails was 16% longer ($\sim$ 1.0 cm) and 12% wider ($\sim$ 0.4 cm) than the shell of Control snails, and they were 43% heavier ($\sim$ 13 mg). The shell of snails exposed once during their post-embryonic development (Early, Middle, Late) was 7% longer ($\sim$ 0.4 cm) and 5% wider ($\sim$ 0.2 cm) than the shell of Control snails, and they were 19% heavier ($\sim$ 6 mg). There was no significant difference in size between snails exposed during all their post-embryonic development (Lifelong) and unexposed snails.

Shell thickness (without and with correction by snail size; Figure 3D-E; Table 1D-E) was influenced by developmental exposure to predator cues. All snails exposed to predator cues had a thicker shell than unexposed snails. Investigating further, there was no significant difference in shell thickness between snails from all exposure windows (Embryo, Early, Middle, Late, Lifelong; Table 1D; Figure 5). Exposed snails had a 31% thicker shell ($\sim$ 0.04 mm) than unexposed snails.

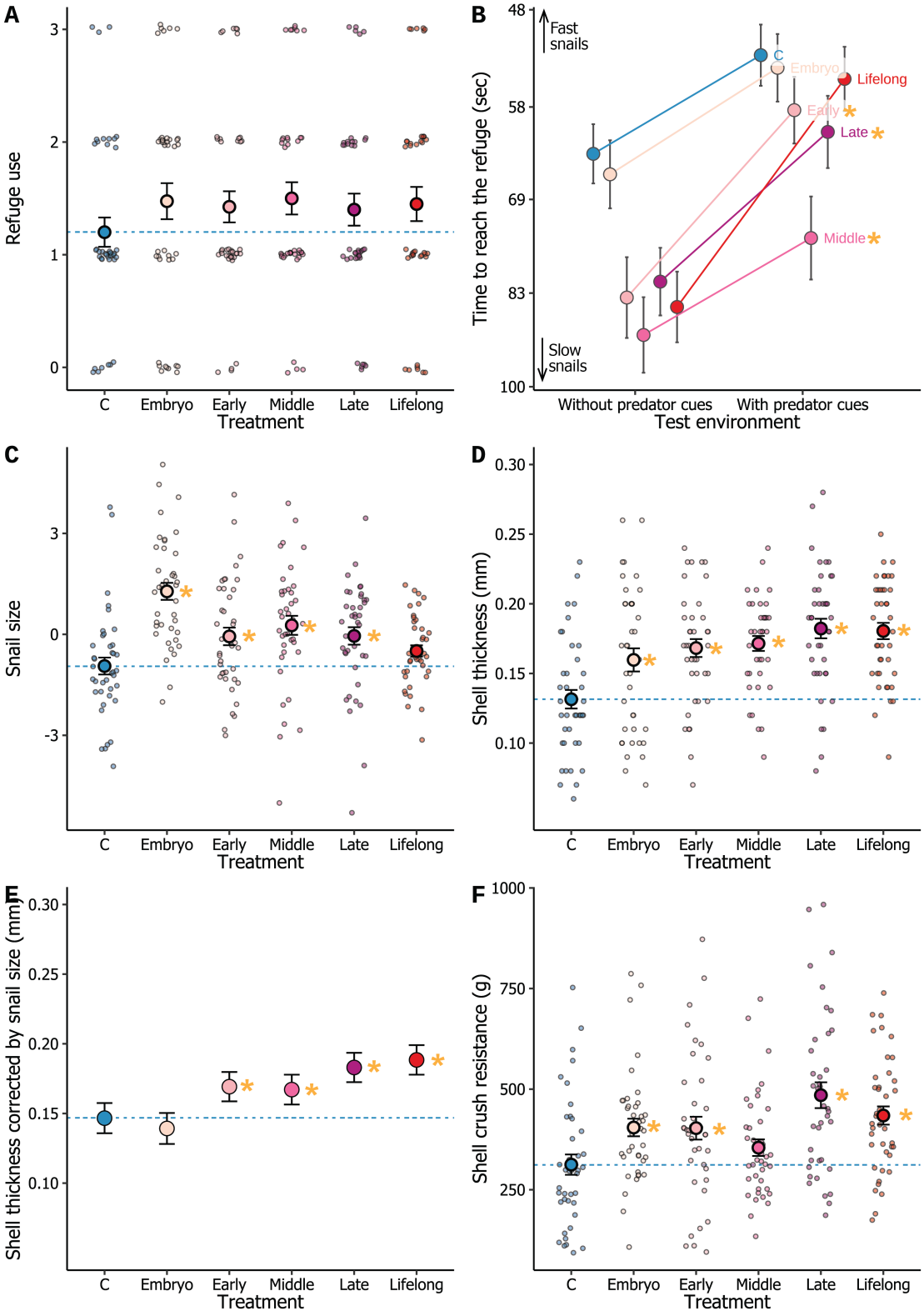

Figure 3 – Effect of exposure at different developmental windows on the WGP of inducible defences. Results of the F1 generation for **A**- refuge use (= number of times a snail was out the water out of four trials), **B**- time to reach the refuge zone (= time to crawl out of the water), **C**- snail size (= first axis of a PCA on shell length, shell width and snail mass), **D**- shell thickness, **E**- shell thickness independent of snail size, and **F**- shell crush resistance. Small dots are raw data for each individual and big dots are means with their standard error; except for plot E where big dots are model predicted means with their 95% CI. Orange asterisks indicate treatments significantly different from the Control treatment (see tests of parameter estimates in Table 1).

Table 1 – Effect of exposure at different developmental windows on the WGP of inducible defences. For all F1 snail defences, the 1st table 'Global model test' shows the effect size and statistical test of the fixed effect(s). In particular, the fixed effect 'Treatment' tests the effect of exposure at different developmental windows on the defence expression level. The 2nd table 'Tests of parameter estimates' shows estimates of the linear model parameters and their t-test, allowing to compare the defence expression level of each treatment with the Control treatment (which is the intercept), but only if there is a significant effect of Treatment. The 3rd table 'Custom pairwise contrasts' shows the custom pairwise contrasts among the treatments for which the defence expression level was significantly different from the Control treatment to test for the presence of sensitive windows. **A**- Generalised linear model on refuge use (= number of times a snail was out of the water out of four trials). **B**- Linear mixed model on time to reach the refuge zone (= time to crawl out of the water) including also results of the random effect. **C**- Linear model on snail size (= first axis of a PCA on shell length, shell width and snail mass). **D**- Linear model on shell thickness. **E**- Linear model on shell thickness including snail size as a covariate. **F**- Linear model on shell crush resistance. SE = standard error. df = degree of freedom. Numdf, Dendf = degree of freedom of the numerator and denominator of the F-statistic. P = p-value.

A. Refuge use

Global model test

Fixed effect	R^2	χ^2	df	P
Treatment	0.01	2.60	5, N =240	0.7613

B. Time to reach the refuge

Global model test

Fixed effect	η^2	F	Numdf, Dendf	P
Treatment	0.08	4.16	5, 234	0.0012*
Test environment	0.08	66.10	1, 718	1.9e-15*
Repetition	0.00	1.24	1, 718	0.2650
Random effect	**Variance**	**χ^2**	**df**	**P**
Snail identity	0.028	112.48	1	3.1e-15*

Tests of parameter estimates

Parameter	Estimate (SE)	t	df	P
(Intercept)	-1.83 (0.029)	-62.37	308	1.2e-176*
Embryo	-0.01 (0.039)	-0.36	234	0.7179
Early	-0.08 (0.039)	-2.17	234	0.0310*
Middle	-0.15 (0.039)	-3.98	234	9.3e-05*
Late	-0.09 (0.039)	-2.24	234	0.0263*
Lifelong	-0.07 (0.039)	-1.93	234	0.0545
Test env. with predator cues	0.12 (0.015)	8.13	718	1.9e-15*
#2 Repetition	0.02 (0.015)	1.12	718	0.2650

Custom pairwise contrasts

Treatment1	Treatment2	Difference (SE)	t	df	P
Early	Middle	0.07 (0.04)	1.81	234	0.3607
Early	Late	0.00 (0.04)	0.06	234	1.0000
Middle	Late	-0.07 (0.04)	-1.74	234	0.3607

C. Snail size

Global model test

Fixed effect	η^2	F	Numdf, Dendf	P
Treatment	0.16	9.19	5, 234	5.3e-08*

Tests of parameter estimates

Parameter	Estimate (SE)	t	df	P
(Intercept)	-0.94 (0.249)	-3.79	234	0.0002*
Embryo	2.22 (0.351)	6.31	234	1.4e-09*
Early	0.88 (0.351)	2.50	234	0.0130*
Middle	1.21 (0.351)	3.44	234	0.0007*
Late	0.89 (0.351)	2.54	234	0.0116*
Lifelong	0.45 (0.351)	1.27	234	0.2041

Custom pairwise contrasts

Treatment1	Treatment2	Difference (SE)	t	df	P
Embryo	Early	1.34 (0.35)	3.81	234	0.0011*
Embryo	Middle	1.01 (0.35)	2.87	234	0.0179*
Embryo	Late	1.32 (0.35)	3.77	234	0.0011*
Early	Middle	-0.33 (0.35)	-0.94	234	1.0000
Early	Late	-0.01 (0.35)	-0.04	234	1.0000
Middle	Late	0.32 (0.35)	0.90	234	1.0000

D. Shell thickness

Global model test

Fixed effect	η^2	F	Numdf, Dendf	P
Treatment	0.14	7.81	5, 233	8.1e-07*

Tests of parameter estimates

Parameter	Estimate (SE)	t	df	P
(Intercept)	0.13 (0.007)	19.72	233	1.5e-51*
Embryo	0.03 (0.009)	3.00	233	0.0030*
Early	0.04 (0.009)	3.90	233	0.0001*
Middle	0.04 (0.009)	4.22	233	3.5e-05*
Late	0.05 (0.009)	5.38	233	1.8e-07*
Lifelong	0.05 (0.009)	5.19	233	4.5e-07*

Custom pairwise contrasts

Treatment1	Treatment2	Difference (SE)	t	df	P
Embryo	Early	-0.01 (0.01)	-0.90	233	1.0000
Embryo	Middle	-0.01 (0.01)	-1.24	233	1.0000
Embryo	Late	-0.02 (0.01)	-2.39	233	0.1786
Embryo	Lifelong	-0.02 (0.01)	-2.20	233	0.2592
Early	Middle	-0.00 (0.01)	-0.35	233	1.0000
Early	Late	-0.01 (0.01)	-1.48	233	1.0000
Early	Lifelong	-0.01 (0.01)	-1.30	233	1.0000
Middle	Late	-0.01 (0.01)	-1.13	233	1.0000
Middle	Lifelong	-0.01 (0.01)	-0.94	233	1.0000
Late	Lifelong	0.00 (0.01)	0.19	233	1.0000

E. Shell thickness corrected by snail size

Global model test

Fixed effect	η^2	F	Numdf, Dendf	P
Treatment	0.22	12.15	5, 232	1.8e-10*
Snail size	0.36	130.44	1, 232	2.9e-24*

Tests of parameter estimates

Parameter	Estimate (SE)	t	df	P
(Intercept)	0.15 (0.006)	26.62	232	1.9e-72*
Embryo	-0.01 (0.008)	-0.90	232	0.3671
Early	0.02 (0.008)	2.95	232	0.0035*
Middle	0.02 (0.008)	2.63	232	0.0092*
Late	0.04 (0.008)	4.75	232	3.6e-06*
Lifelong	0.04 (0.008)	5.51	232	9.6e-08*
Snail size	0.02 (0.001)	11.42	232	2.9e-24*

Custom pairwise contrasts

Treatment1	Treatment2	Difference (SE)	t	df	P
Early	Middle	0.00 (0.01)	0.28	232	0.9507
Early	Late	-0.01 (0.01)	-1.82	232	0.2097
Early	Lifelong	-0.02 (0.01)	-2.53	232	0.0603
Middle	Late	-0.02 (0.01)	-2.08	232	0.1531
Middle	Lifelong	-0.02 (0.01)	-2.77	232	0.0361*
Late	Lifelong	-0.01 (0.01)	-0.71	232	0.9507

F. Shell crush resistance

Global model test

Fixed effect	η^2	F	Numdf, Dendf	P
Treatment	0.11	5.59	5, 232	6.9e-05*

Tests of parameter estimates

Parameter	Estimate (SE)	t	df	P
(Intercept)	312.50 (25.317)	12.34	232	3.1e-27*
Embryo	92.21 (35.803)	2.58	232	0.0106*
Early	9 0.63 (35.803)	2.53	232	0.0120*
Middle	42.12 (36.032)	1.17	232	0.2436
Late	172.53 (36.032)	4.79	232	3.0e-06*
Lifelong	121.96 (35.803)	3.41	232	0.0008*

Custom pairwise contrasts

Treatment1	Treatment2	Difference (SE)	t	df	P
Embryo	Early	1.58 (35.80)	0.04	232	1.0000
Embryo	Late	-80.32 (36.03)	-2.23	232	0.1437
Embryo	Lifelong	-29.75 (35.80)	-0.83	232	1.0000
Early	Late	-81.90 (36.03)	-2.27	232	0.1437
Early	Lifelong	-31.32 (35.80)	-0.87	232	1.0000
Late	Lifelong	50.57 (36.03)	1.40	232	0.6472

After correction for snail size, only snails exposed during their post-embryonic development (Early, Middle, Late, Lifelong) had a thicker shell than unexposed snails: their shells were 21% thicker (∼0.03 mm) compared to shells from unexposed snails of the same size. Investigating further, Middle snails had a thicker shell than Lifelong snails relative to their size, but there was no other significant difference between snails from Early, Middle, Late and Lifelong windows (Table 1E; Figure 5).

Shell crush resistance (Figure 3F; Table 1F) was influenced by developmental exposure to predator cues. Besides snails exposed during mid post-embryonic development (Middle), all snails exposed to predator cues had their shell harder to crush than unexposed snails. Investigating further, there was no significant difference in shell crush resistance between snails from Embryo, Early, Late and Lifelong windows (Table 1F; Figure 5). Their shells needed 431 g of force to crush while those of unexposed snails needed 312 g of force (∼ 38% higher).

2. How did the exposure at different developmental windows affect TGP?

Refuge use (Figure 4A; Table 2A) was influenced by parental exposure to predator cues. Offspring of parents exposed to predator cues used the refuge zone out of the water more often than offspring from unexposed parents (who received the Control treatment) regardless of the window of parental exposure. Investigating further, there was no significant difference in refuge use between offspring from all parental exposure windows (Embryo, Early, Middle, Late, Lifelong; Table 2A; Figure 5).

Time to reach the refuge (Figure 4B; Table 2B) was influenced by test environment: offspring reached the refuge zone on average 19 sec faster when they were tested in an environment with predator cues than without predator cues, similarly to their parents. However, there was no evidence that time to reach the refuge was influenced by parental exposure to predator cues.

Snail size (Figure 4C; Table 2C) was influenced by parental exposure to predator cues. Offspring of parents who received the Embryo, Middle, and Lifelong treatments were larger than offspring of unexposed parents: their shell was 6% longer (∼ 0.4 cm) and 7% wider (∼ 0.3 cm), and they were 21% heavier (∼ 9 mg). Investigating further, offspring of Embryo parents were larger than offspring of Lifelong parents, but there was no other significant difference in size between offspring of Embryo, Middle and Lifelong parents (Table 2C; Figure 5). There was no evidence that offspring of parents who received the Early and Late treatments were different in size from offspring of unexposed parents.

Shell thickness (without and with correction by snail size; Figure 4D-E; Table 2D-E) was influenced by parental exposure to predator cues. Only offspring of parents who received the Middle treatment had a thicker shell than offspring of unexposed parents: their shells were 12% thicker (∼ 0.02 mm) (Figure 5). There was no evidence that offspring of parents from the other treatments had thicker shells than offspring of unexposed parents. After correction for snail size, there was no significant difference of offspring shell thickness between the Control and any other treatment, despite a significant global effect of treatment.

Shell crush resistance (Figure 4F; Table 2F) was influenced by parental exposure to predator cues. Offspring of parents who received the Embryo and Middle treatments (marginal effect for the Lifelong treatment) had their shell significantly harder to crush than offspring of unexposed parents. Investigating further, there was no signicant difference in shell crush resistance between offspring of Embryo and Middle parents (Table 2F; Figure 5). Their shell needed 390 g of force to crush while that of offspring from unexposed parents needed 321 g of force (∼ 22% higher). In contrast, offspring of parents who received the Late treatment had their shell easier to crush than offspring of unexposed parents: their shell needed 256 g of force to crush (∼ 20% lower).

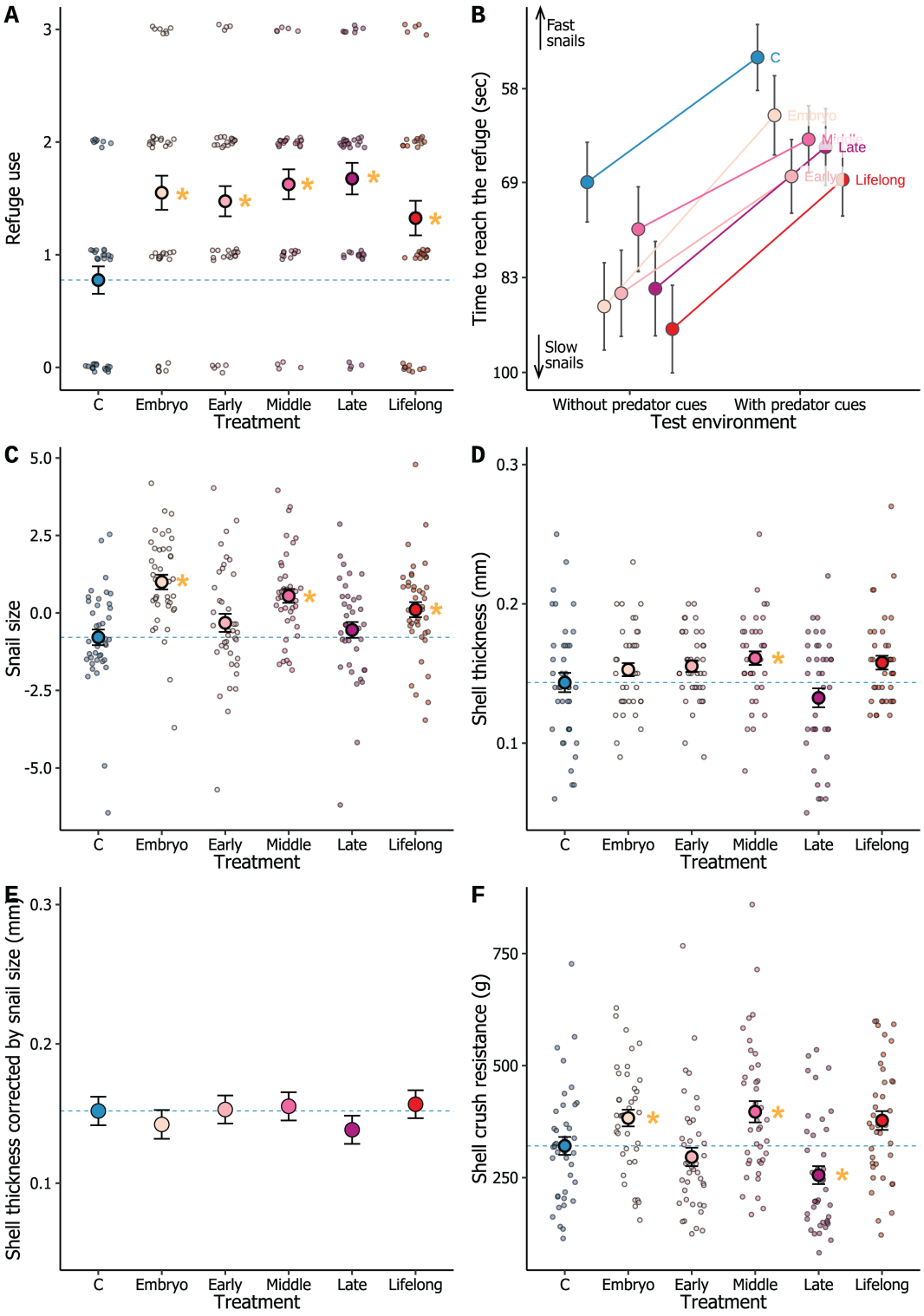

Figure 4 – Effect of exposure at different developmental windows on the TGP of inducible defences. Results of the F2 generation for **A**- refuge use (= number of times a snail was out the water out of three trials), **B**- time to reach the refuge zone (= time to crawl out of the water), **C**- snail size (= first dimension of a PCA on shell length, shell width and snail mass), **D**- shell thickness, **E**- shell thickness independent of snail size, and **F**- shell crush resistance. As a reminder, parental F1 snails underwent the different treatments (Control, Embryo, etc) but offspring F2 snails were only raised in control water without predator cues, see Figure 1. Small dots are raw data for each offspring and big dots are means with their standard error; except for plot E where big dots are model predicted means with their 95%CI. Orange asterisks indicate treatments significantly different from the Control treatment (see tests of parameter estimates in Table 2).

Table 2 – Effect of exposure at different developmental windows on the TGP of inducible defences. For all F2 snail defences, the 1st table 'Global model test' shows the effect size and statistical test of the fixed effect(s). In particular, the fixed effect 'Treatment' tests the effect of parental exposure at different developmental windows on the defence expression level of offspring. The 2nd table 'Tests of parameter estimates' shows estimates of the linear model parameters and their t-test, allowing to compare the defence expression level of each treatment with the Control treatment (which is the intercept), but only if there is a significant effect of Treatment. The 3rd table 'Custom pairwise contrasts' shows the custom pairwise contrasts among the treatments for which the defence expression level was significantly different from the Control treatment to test for the presence of sensitive windows. **A**- Generalised linear model on refuge use (= number of times a snail was out of the water out of three trials). **B**- Linear mixed model on time to reach the refuge zone (= time to crawl out of the water) including also results of the random effect. **C**- Linear model on snail size (= first axis of a PCA on shell length, shell width and snail mass). **D**- Linear model on shell thickness. **E**- Linear model on shell thickness including snail size as a covariate. **F**- Linear model on shell crush resistance. SE = standard error. df = degree of freedom. Numdf, Dendf = degree of freedom of the numerator and denominator of the F-statistic. P = p-value.

A. Refuge use

Global model test

Fixed effect	R^2	χ^2	df	P
Treatment	0.09	30.58	5, N =240	1.1e-05*

Tests of parameter estimates

Parameter	Estimate (SE)	z	P
(Intercept)	-1.05 (0.209)	-5.06	4.3e-07*
Embryo	1.12 (0.277)	4.04	5.2e-05*
Early	1.02 (0.277)	3.68	0.0002*
Middle	1.22 (0.278)	4.40	1.1e-05*
Late	1.29 (0.278)	4.64	3.5e-06*
Lifelong	0.82 (0.278)	2.95	0.0032*

Custom pairwise contrasts

Treatment1	Treatment2	odds.ratio (SE)	z	P
Embryo	Early	1.11 (0.29)	0.39	1.0000
Embryo	Middle	0.90 (0.23)	-0.39	1.0000
Embryo	Late	0.85 (0.22)	-0.65	1.0000
Embryo	Lifelong	1.35 (0.35)	1.16	1.0000
Early	Middle	0.82 (0.21)	-0.77	1.0000
Early	Late	0.77 (0.20)	-1.03	1.0000
Early	Lifelong	1.22 (0.32)	0.78	1.0000
Middle	Late	0.93 (0.24)	-0.26	1.0000
Middle	Lifelong	1.49 (0.39)	1.55	1.0000
Late	Lifelong	1.60 (0.42)	1.80	0.7135

B. Time to reach the refuge

Global model test

Fixed effect	η^2	F	Numdf, Dendf	P
Treatment	0.03	1.44	5, 234	0.2109
Test environment	0.07	50.60	1, 718	2.7e-12*
Repetition	0.01	10.80	1, 718	0.0011*
Random effect	**Variance**	**χ^2**	**df**	**P**
Snail identity	0.016	62.22	1	< 0.0001*

C. Snail size

Global model test

Fixed effect	η^2	F	Numdf, Dendf	P
Treatment	0.14	7.35	5, 234	2.0e-06*

Tests of parameter estimates

Parameter	Estimate (SE)	t	df	P
(Intercept)	-0.78 (0.251)	-3.12	234	0.0020*
Embryo	1.78 (0.355)	5.01	234	1.1e-06*
Early	0.47 (0.355)	1.31	234	0.1910
Middle	1.33 (0.355)	3.75	234	0.0002*
Late	0.24 (0.355)	0.67	234	0.5034
Lifelong	0.89 (0.355)	2.50	234	0.0129*

Custom pairwise contrasts

Treatment1	Treatment2	Difference (SE)	t	df	P
Embryo	Middle	0.45 (0.36)	1.26	234	0.4182
Embryo	Lifelong	0.89 (0.36)	2.51	234	0.0386*
Middle	Lifelong	0.44 (0.36)	1.25	234	0.4182

D. Shell thickness

Global model test

Fixed effect	η^2	F	Numdf, Dendf	P
Treatment	0.07	3.26	5, 234	0.0072*

Tests of parameter estimates

Parameter	Estimate (SE)	t	df	P
(Intercept)	0.14 (0.006)	25.04	234	4.0e-68*
Embryo	0.01 (0.008)	1.14	234	0.2550
Early	0.01 (0.008)	0.74	234	0.4699
Middle	0.02 (0.008)	2.16	234	0.0319*
Late	-0.01 (0.008)	-1.36	234	0.1761
Lifelong	0.01 (0.008)	1.76	234	0.0801

E. Shell thickness corrected by snail size

Global model test

Fixed effect	η^2	F	Numdf, Dendf	P
Treatment	0.04	4.15	5, 233	0.0013*
Snail size	0.22	64.52	1, 233	4.7e-14*

Tests of parameter estimates

Parameter	Estimate (SE)	t	df	P
(Intercept)	0.15 (0.005)	29.27	233	5.4e-80*
Embryo	-0.01 (0.008)	-1.28	233	0.2026
Early	0.00 (0.007)	0.15	233	0.8845
Middle	0.00 (0.007)	0.45	233	0.6524
Late	-0.01 (0.007)	-1.88	233	0.0613
Lifelong	0.00 (0.007)	0.66	233	0.5109
Snail size	0.01 (0.001)	8.03	233	4.7e-14*

F. Shell crush resistance

Global model test

Fixed effect	η^2	F	Numdf, Dendf	P
Treatment	0.14	7.44	5, 233	1.7e-06*

Tests of parameter estimates

Parameter	Estimate (SE)	t	df	P
(Intercept)	320.88 (20.653)	15.54	233	7.8e-38*
Embryo	62.10 (29.207)	2.13	233	0.0345*
Early	-24.73 (29.207)	-0.85	233	0.3980
Middle	76.22 (29.207)	2.61	233	0.0096*
Late	-65.26 (29.207)	-2.23	233	0.0264*
Lifelong	56.65 (29.394)	1.93	233	0.0551

Custom pairwise contrasts

Treatment1	Treatment2	Difference (SE)	df	t	P
Embryo	Middle	-14.12 (29.21)	-0.48	233	1.0000
Embryo	Late	127.36 (29.21)	4.36	233	<0.0001*
Middle	Late	141.48 (29.21)	4.84	233	<0.0001*

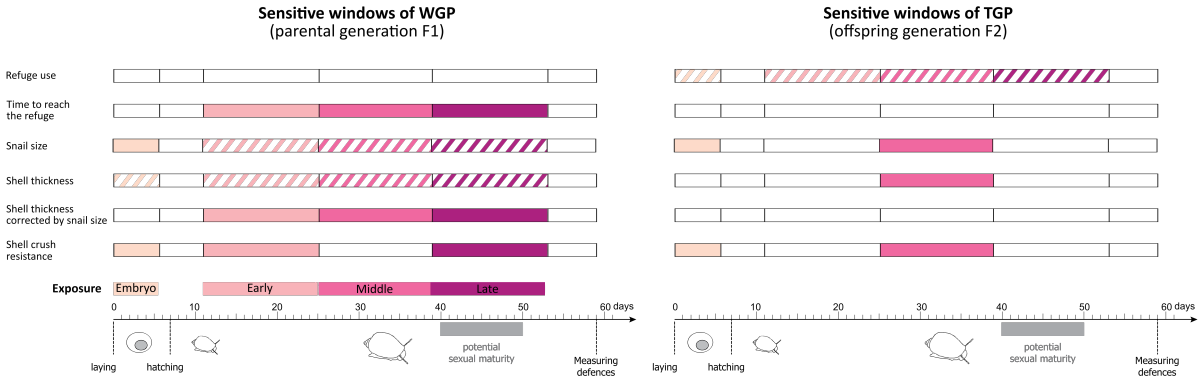

Figure 5 – Summary figure. For each defence, the exposure window is filled with colour when it was a sensitive window of WGP (left) or TGP (right). The exposure window is stripped when it was not a sensitive window but nevertheless induced a higher expression level of that defence.

Discussion

We studied how exposure to predator cues at different developmental windows influenced WGP and TGP of defences in prey. We assessed defences expressed in adult parents and adult offspring of the snail *Physa acuta* after having exposed parents to cues of crayfish presence at different developmental windows (embryonic development, early, middle, or late post-embryonic development). We demonstrated the presence of sensitive windows of WGP and TGP depending on the defence considered. Our results confirmed that early-life periods of development (embryonic and early post-embryonic) are sensitive windows of WGP. However, later developmental periods were also sensitive. There were fewer sensitive windows of TGP: only the embryonic and/or the mid post-embryonic developmental window. When the embryonic period was a sensitive window of WGP for a defence, it was also a sensitive window of TGP for that defence, which partly supports our first prediction that sensitive windows of TGP are shared with WGP. However, the late development was never a sensitive window of TGP contrary to our second prediction. In what follows, we provide a more detailed description of the sensitive windows and whether or not they are compatible with the theory and possible reasons for this.

1. Sensitive windows of WGP

Our results provide evidence of different sensitive windows of WGP depending on the defence considered. Concerning the behavioural defences, all the post-embryonic development (from Early to Late) was a sensitive window inducing snails to reach the refuge zone more slowly (the embryonic window had no effect). While none of the exposure windows significantly induced snails to use the refuge zone out of the water. Concerning the morphological defences, snails exposed during embryonic development were larger than snails exposed during post-embryonic development and the latter were larger than unexposed snails. This suggests that the embryonic window is the sensitive window for the induction of larger size, although later exposure can also influence snail size. There was no sensitive window for the induction of a thicker shell as all exposed snails had an equally thicker shell than unexposed snails. However, after removing the effect of snail size on shell thickness, only snails exposed during post-embryonic development (from Early to Late) had thicker shells than unexposed snails. None of the exposure windows stood out from the others, suggesting the sensitive window for the induction of shell thickening is all the post-embryonic development. The results also provide evidence that there were several sensitive windows (Embryo, Early and Late) that induced higher resistance of shells against crush. The results thus demonstrated that the developmental window of cue exposure is an important factor driving anti-predator within-generational responses in line with the literature in other species (Hoverman and Rick A. Relyea, 2007; Mikulski et al., 2005; Rick A. Relyea, 2003; Weiss et al., 2016). Our results confirmed our prediction that early stages are highly sensitive to the environment, here *P. acuta* embryos are able to detect predator cues, but also highlighted that the later stages are sensitive. The onset of sensitive periods is predicted

to start as soon as when cues become available to individuals (Judy Ann Stamps and Luttbeg, 2022); it is thus predicted that embryo snails can detect olfactory cues of crayfish as embryos develop in the water. It may be however surprising that they can detect them before their sensory organs are fully developed. In our experiment, snails were exposed during almost the entire embryonic development and we suspect that they detected predator cues only at the end of embryonic development when snails are fully developed in their eggs, just before hatching. It would be interesting to partition exposure during embryonic development to test this hypothesis.

Our results confirmed that early-life development is a sensitive window of WGP, but only for snail size. There was no sensitive window for shell thickness, the sensitive window was the post-embryonic development for the shell thickening (i.e. shell thickness corrected by snail size) and time to reach the refuge, and the early-life and late post-embryonic development were sensitive windows for shell crush resistance. The different sensitive windows depending on the defence could be explained by the existence of two defensive phenotypes: 1/ a very large size when exposure to predator cues occurred at the embryonic stage, and 2/ a large size and shell thickening when exposure occurred at post-embryonic stages. The second defensive phenotype is associated with a decreased escape behaviour (i.e. decreased time to reach the refuge) suggesting a trade-off between behavioural and morphological defences with snails not afraid of facing the predator as morphologically defended or not able to accumulate the costs of producing shell material and escaping fastly (Ahlgren et al., 2015; Steiner and Van Buskirk, 2008). We do not detect however such trade-offs in our data (see Supplementary Information 3). Alternatively, this decreased escape response could also result from the negative carry-over effect of the long exposure of these snails (14 days) to the potentially stressful predator cues. Early exposure to predators may have directed snails to a developmental pathway of high growth rate, while post-embryonic exposures may have been too late to direct snails towards the highly accelerated growth pathway and forced snails to mineralise their shell to increase its thickness and therefore its resistance. A differential defensive phenotype depending on the stage exposed to predator cues has also been described in *Hyla versicolor* tadpoles: tadpoles exposed at an early juvenile stage based their defensive phenotype on tail shape and behaviour, whereas tadpoles exposed at a late juvenile stage based their defensive phenotype on size and shape of the tail and body (Rick A. Relyea, 2003). Each defensive phenotype may depend on the constraints, benefits and costs associated with the defence induction at each developmental period.

Theoretical models can predict no sensitive window or sensitive window until late development for specific combinations of parameter values. First, they can predict an absence of sensitive window if the plasticity is only based on and linearly linked to the informational state of individuals, i.e. how many cues about the presence of predators individuals have gathered (see the Window protocol with ABI variant in Stamps 2022 which is our protocol). This is not the case as we should have observed otherwise that snails in the Lifelong exposure express the highest level of defence (as they have been exposed 42 days to predator cues in total), followed by snails in the Early, Middle and Late exposure (14 days of exposure to predator cues), and by snails in the Embryo exposure (5 days of exposure). Second, a sensitive window until late development is predicted if individuals remain uncertain about their environment across development (Judy A. Stamps and Krishnan, 2017). A first possibility is that they have been raised in the absence of any informative cues (Judy A. Stamps and Krishnan, 2017). It seems not the case in our study as the absence of predator cues must be informative about the density of predators, so even snails in the late treatment had access to informative cues about the true absence of predators before being exposed to predator cues. A second possibility is when individuals have a strong initial belief about the likely state of their environment (belief = prior distribution in Bayesian models modelling the prior knowledge of individuals at the beginning of their development about the true state of the environment transmitted by parents through genes, transgenerational plasticity or genetic parental effects; English et al., 2016; Judy A. Stamps and Krishnan, 2014, 2017). Snails could have a strong belief that there are predators in their environment because they may have evolved for many generations in the presence of predators (see next section 'sensitive windows TGP'). They thus remain unsure about the true presence of predators even if they detect no predator cues until very late during development and maintain their capacity for inducing

defences in response to predator cues. This could be emphasised if the absence of predator cues is not very reliable about the true absence of predators (Panchanathan and Frankenhuis, 2016). The strong belief in the presence of predators could also explain why snails in the Embryo treatment developed defences at adult stage even after only 5 days of exposure during the embryonic development (when prior knowledge and early environmental cues agree, individuals are confident about their true environment and hence less likely to change their belief when exposed to no predator cues afterwards). This hypothesis could be tested by comparing the sensitive windows of different populations of snails that have evolved with or without the presence of crayfish for many generations. Finally, a possibility that we proposed is a differential reliability of the different cues (detecting predator cues may be more reliable about the true presence of predators, than detecting no predator cues about the true absence of predators) combined with a differential fitness loss when the phenotype mismatches the true environmental state. Predicting an absence of predators when predators are present should have more negative fitness consequences as individuals don't develop defences and should suffer high mortality from predation, than predicting the presence of predators when predators are absent as the costs are only the costs of producing unnecessary defences. This scenario should select for high sensitivity to predator cues across development and thus no sensitive window or sensitive window until late development.

2. Sensitive windows of TGP

As we observed for WGP, our results demonstrated the existence of sensitive windows for TGP depending on the defence considered. Concerning the behavioural defences, all parental exposure windows induced a more frequent but similar refuge use in offspring; there was thus no apparent sensitive window for this defence. In contrast, no parental exposure window induced a significant change in time to reach the refuge. Concerning the morphological defences, the offspring from parents exposed during the embryonic and mid post-embryonic windows were larger, suggesting the existence of two sensitive windows for TGP on growth. The mid post-embryonic exposure was a sensitive window for the induction of a thicker shell. However, when shell thickness is corrected for size, there was no longer any influence of parental exposure indicating that the sensitive window for shell thickness previously described was a consequence of the change in growth induced during the mid post-embryonic window. Embryonic and mid post-embryonic exposure were also two sensitive windows inducing a more crush-resistant shell in offspring, which is consistent with the sensitive windows triggering a change in snail size. A more surprising result, and difficult to explain, is that the late post-embryonic exposure led to a less crush-resistant shell in offspring, which is nevertheless consistent with the smaller size and shell thickness of these offspring (but non-significant results for size and shell thickness). This study adds a demonstration that the exposure window is an important factor driving TGP responses in addition to the study of Mikulski et al. (2010; Daphnia life-history defences) and studies in other systems (e.g. cyanobacteria exposure in Radersma et al. 2018; heat stress in Deng et al. 2021).

Interestingly, the embryonic period was a sensitive window for snail size and crush resistance for both WGP and TGP, which is partly in line with our prediction that WGP and TGP share the same sensitive windows. These results suggest that WGP and TGP are two processes mechanically linked. The embryonic period that greatly impacts the parental phenotype may have had carry-over impacts on offspring phenotype (via state-based or information-based TGP) and/or they may share common molecular mechanisms to detect environmental cues and induce the according phenotype (Stein et al., 2018). The latest meta-analyses on TGP (Yin et al., 2019; Zhang et al., 2020) and the only other experimental study on sensitive TGP windows of anti-predator defences (Mikulski and Pijanowska, 2010) support that sensitive windows of TGP depend on those of WGP. The latter showed that *Daphnia* exposed to predator cues during the 4st instar developmental window had the greatest change in their anti-predator life-history responses, and also had the most significant influence on those of their offspring. However, we have also highlighted that the mid post-embryonic development was a sensitive window for TGP, which is not

in line with the hypothesis of shared sensitive windows. When the mid post-embryonic development was a sensitive window of TGP for a defence, it was not a sensitive window of WGP for that defence. This suggests that TGP, although linked to WGP, may also be induced via a specific channel, independent of WGP induction and expression, and reflect the detection and transmission of environmental information through generations (information-based TGP). This echoes the molecular studies which find a core set of similarly expressed genes for WGP and TGP (i.e. same genes in parents and offspring that are differently expressed following exposure of parents to contrasted environments; Stein et al., 2018) or, at the opposite, differential gene expression patterns for WGP and TGP (Hales et al., 2017). When individuals have been exposed all their lives to the same environmental cues (as is the case in most experimental studies), the resulting TGP may thus be a complex combination of the effects of the parental environment on offspring phenotype specific to each developmental window. The effects could sum up when they take different channels or interact with each other when these effects take the same channel in additive or non-additive ways generating hardly predictable patterns. We indeed observed in our study that lifelong parental exposure induced either the same level of defence in offspring as when parents were exposed only during the sensitive window (shell thickness is similar for middle exposure and lifelong exposure) or lifelong exposure induced a different pattern (snail size is larger for the embryo exposure than lifelong exposure, shell crush resistance is influenced by exposure at sensitive windows but not by lifelong exposure).

Another interesting result of our study is that the late developmental window of parents was not a sensitive window for defense expression in offspring. This contradicts our prediction that the higher reliability of late-perceived parental cues should select for late developmental windows of TGP (Bell and Hellmann, 2019; Donelan et al., 2020; Tariel et al., 2020b). This prediction is based on the assumption that the reliability of parental cues depends on the developmental window at which they were perceived during parental development. The validity of this assumption likely depends on the model system and in particular on the rate of environmental change in relation to the generation time. In short-generation time species, several generations are likely to experience the same environmental conditions, and cues perceived during development, whatever the period, are therefore all good predictors of future selective pressures. We strongly suspect that this is the case in our crayfish-snail system as the generation time of *P. acuta* is ca. two months while a crayfish like *F. limosus* can live ca. three years and has a relatively sedentary lifestyle. Consequently, parental cues are certainly reliable about crayfish presence over a period of time well beyond the snail generation time. We suppose this is the same thing for *Daphnia* where late developmental periods are not sensitive windows (Mikulski and Pijanowska, 2010). Considering these aspects reveal that the lack of support for the high sensitivity of late developmental periods is probably due to the focus on short-generation time species to study TGP. Although it seems difficult to realise TGP experiments on long-generation time species, a promising avenue of research to test theoretical predictions about sensitive windows is experimental evolution where the rate of environmental change is manipulated in different lines of a short-generation time species.

3. Conclusion

Looking at the global induced defensive phenotype, exposure at each developmental window, from the embryonic to the late post-embryonic window, has induced defences in adult parents and offspring snails. *P. acuta* snails keep all their life the capacity of inducing defences in response to predator-cue detection, which is in line with the literature in other species (WGP: Hoverman and Rick A. Relyea, 2007; Rick A. Relyea, 2003; Weiss et al., 2016) (TGP: Mikulski and Pijanowska, 2010). In these species, predation must be such a strong selective pressure at all stages of development that it selects prey to retain their ability to within- and trans-generationally induce defences in response to predator cues throughout development as expressing no defence in the presence of predators should cause a large loss of fitness (Hoverman and Rick A. Relyea, 2007; Judy Ann Stamps and Luttbeg, 2022). The developmental window at which predator cues are perceived does not seem to constrain the adaptive potential of WGP and TGP. At the scale of the defensive trait, we demonstrated the existence of sensitive developmental windows of WGP

and TGP for most defensive traits. Developmental windows at which an environment is experienced is thus an important factor driving anti-predator within-and trans-generational responses. Exposure window adds to the many factors that can influence TGP patterns (e.g. parental sex exposed to environmental cues, offspring sex, interactive effects of grand-parental, parental and offspring environments; Bell and Hellmann, 2019; Tariel et al., 2020a; Yin et al., 2019; Zhang et al., 2020) making it difficult to study TGP and determine its adaptive potential. Our results add indeed to the argument that the TGP patterns we often try to interpret as the results of a single process might more likely result from a combination of several processes and underlying mechanisms.

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Conflict of interest disclosure

The authors declare that they comply with the PCI rule of having no financial conflicts of interest in relation to the content of the article.

Data, script and code availability

Data and R code are available online on the OSF project (Tariel-Adam et al., 2022, https://doi.org/10.17605/osf.io/w2cxa).

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Supplementary Information

This supplementary information provides details on:

(1) The rearing of snails during the experiment.
(2) The mating of snails during the experiment and the reproductive system of *Physa acuta*.
(3) Trade-offs between morphological and behavioural defenses.

1. Rearing

Snails were first reared with their siblings in small 70-mL boxes each containing a F1/F2 family. Snails were then reared in medium 300-mL boxes and mixed among families to avoid competition (when we started the Middle treatment). To do this, we randomly placed snails from the different families (but from the same treatment) into the medium boxes until the boxes reached the same density (23-30 individuals per box depending on treatment). 14 days later (when we started the Late treatment), snails were moved to large 1.2-L boxes and again randomly mixed (within the same treatment) into the different boxes until they reached the same density (18-20 individuals per box). We started to observe egg masses in the rearing boxes indicating that some snails reached sexual maturity. Snails continued to develop until reaching a sufficient size to measure their defences. Then 40 snails per treatment were isolated in the small boxes (240 snails in total).

2. Mating

For each of the first four days of F1 defence measurements, F1's refuge use was scored in the morning, then F1 were put in mass-mating groups of 10 F1 in the afternoon for two hours, and finally back to their individual boxes to lay eggs. These eggs constituted the F2 generation (40 F2 families for each parental treatment). F1 mating groups remained the same during all four days and F1 shells were painted with one of 10 colours of nail polish to keep their identity during mating (shell painting is not associated with a change in life-history traits; Pierre-Yves Henry and Jarne, 2007).

The snail *Physa acuta* is hermaphrodite and can self-fertilise but preferentially outcrosses; Selfing occurs when snails can not find mates (Henry et al., 2005). Two virgin snails put together will immediately start copulating, one acting as a female and the other as a male, and then do the other way round in less than 20 min (personal observations). After copulation, the snail usually lays an egg capsule a few hours later containing 10-50 eggs. It can lay an egg capsule every day.

3. Trade-offs between behavioural and morphological defences

To test for trade-offs between behavioural and morphological defences, we added morphological defences as fixed effects in the linear models of behavioural defences. To reduce the number of morphological variables to add to the models, we realised a Principal Component Analysis with all morphological defences: snail total weight (cube-root transformed), shell length, shell width, shell thickness and shell crush resistance. The coordinates on the 1st and 2nd PCA axis (pc1 and pc2) were extracted and used as fixed effects. We included interactions with the Treatment for both pc1 and pc2. The models are shown below. The interactions were not significant at either generation and were therefore removed from the model.

$$\text{refuge use} \sim \text{Treatment} \times \text{pc1} + \text{Treatment} \times \text{pc2}$$

$$-log10(\text{time to reach the refuge}) \sim \text{Treatment} \times \text{pc1} + \text{Treatment} \times \text{pc2} + \text{Test environment} + \text{Repetition} + (1|\text{Snail identity})$$

The pc1 and pc2 fixed effects were not significant at either generation for refuge use (F1 parental generation, pc1: χ^2 = 2.00 P = 0.1568; pc2: χ^2 = 1.15 P = 0.2832; F2 offspring generation, pc1: χ^2 = 0.14 P = 0.7044; pc2: χ^2 = 0.67 P = 0.4114) and time to reach the refuge (F1 parental generation, pc1: F = 2.46 P = 0.1182; pc2: F = 0.97 P = 0.3265; F2 offspring generation, pc1: F = 0.12 P = 0.7316; pc2: F = 1.36 P = 0.2432).

These results did not indicate trade-offs between greater expression of behavioural defences and greater expression of morphological defences.