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Long-term individual and population effects of multiple stressors, using a model freshwater mollusc (*Lymnaea stagnalis*)

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ABSTRACT

Human activities are driving biodiversity loss by increasing the exposure to multiple environmental stressors with pollution, climate change and invasive species being amongst the most important contributors. Here, we used the pond snail, *Lymnaea stagnalis*, as a model organism to investigate the combined effects of an environmentally relevant pollutant mixture, temperature increase and an invasive predator cue on physiological endpoints and behaviour. Dose-response data from single stressor exposures gathered over the partial life-cycle exposure period were used to calculate effect concentrations (EC10 and EC30) and responses to their combinations were investigated. At the EC30 for temperature (20 °C *vs.* 22 °C), effects were widely observed. While growth and reproduction were stimulated, this modest temperature increase negatively impacted survival (20 % reduction). Neither the pollutant mixture nor the predator cue severely impacted the examined responses and did not exacerbate the temperature effects. By contrast, the application of all three stressors at the EC30 level tended to ameliorate stress, compared to the temperature EC30 alone. Exploratory analyses also revealed that snails that avoided the predator cue by moving above the water line exhibited higher growth than those that remained in the water. Our results indicate that a number of organismal trade-offs may be occurring, such as between survival and reproduction, highlighting the complexities of predicting the impact of multiple stressors. Finally, the pronounced effects observed in response to this temperature increase (+2°C) is concerning, as this is within currently observed planetary warming, with organisms inhabiting small water bodies particularly susceptible due to their small water volumes.

1. Introduction

It has been demonstrated in a wide variety of taxa that population declines are driven by multiple interacting stressors (e.g., insects, Forister et al., 2010; fish, Hecky et al., 2010; amphibians, Hof et al., 2011; birds, Jetz, Wilcove and Dobson, 2007). Freshwater (FW) ecosystems may be particularly vulnerable to multiple stressors, due to their often close proximity to human infrastructure and populations. The infinite number of potential combinations of multiple stressors present logistical and theoretical challenges to study their effects, and predicting their joint effects remains a huge challenge (Birk, 2019; Pirotta et al., 2022). The vast majority of laboratory studies have studied the effects of two stressors over short-term exposures, with mortality the most commonly reported endpoint, and this complicates extrapolation to sublethal individual- and population- level effects that are relevant in the context of ecosystems (reviewed in: Jackson et al., 2016; Nõges et al., 2016; Orr et al., 2024). In ecosystems, organisms are continually exposed to chronic stressor levels over long time periods, with stressors having the potential to impact a whole suite of endpoints, prior to the resultant mortality of the individual. Therefore, the effects of multiple stressors on FW biota are poorly understood and experiments focused on stressor combinations at environmentally relevant levels and combinations (> 3

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stressors and including pollutant mixtures), examining sub-lethal effects on physiological endpoints under chronic, long-term exposures are still missing. Whilst these types of laboratory experiments cannot replicate natural conditions, by better incorporating environmental realism into such experiments, they can contribute to untangling the real effects of multiple stressors on wildlife.

In FW organisms, short-term, partial life-cycle exposures to three stressors have been investigated in *Daphnia*. For example, it was reported that *Daphnia pulex* exposed to different combinations of pyrimethanil (50 μg/L; fungicide), temperature stress (warming) and predator kairomones (Chironomid midge larvae) for 21 days displayed alterations in growth and reproduction; with changes to overall growth rate primarily driven by warming, followed by predator stress (Scherer et al., 2013). A somewhat similar study with *Daphnia magna*, with exposures to warming, nitrate (50 mg/L) and sub-optimal feed for 35 days reported a negative direct effect of heatwaves and a positive direct effect of ntirate, however, the quality of the feed was the most important factor determining survival, growth and reproduction (Akter et al., 2024). Another study in *Daphnia magna* exposed to predator kairomones (Chironomid midge larvae), parasites (obligate endoparasite *Pasteuria ramosa*) and carbaryl (50 μg/L, insecticide) for 21 days reported complex interactions between stressors, encompassing synergism, antagonism and additivity depending on endpoint and combination (authors used Independent Action model: Coors and De Meester, 2008). Apart from in *Daphnia*, to the authors' knowledge, studies that encompass endpoints across an organisms life-cycle and investigating the effects of at least three stressors are not available.

In this study, we continued our previous work on laboratory-based stressor ranking by combining ubiquitous stressors at environmentally relevant levels to investigate long-term effects on survival, growth, reproduction and behaviour, using the model gastropod species, *Lymnaea stagnalis*. Using dose-response data reported by Moore et al. (2021), here we combined stressors at realistic levels and across a range of intensities/combinations over a partial life-cycle exposure in *L. stagnalis*. This species is an OECD test species for chemicals risk assessment, and as such, much is known regarding their husbandry and baseline biology (OECD, 2016). In this study, we investigated the effects of an environmentally relevant pollutant mixture (derived from European FW data; see Moore et al., 2021 for details), applied at levels that elicited 10 % and 30 % effects on survival (i.e. EC10, EC30) and invasive predator cue (EC10, EC30: *Pacifastacus leniusculus*), both in the absence (i.e., control temperature: 20 °C) and the presence of elevated temperature (EC30: 22°C). The EC10 and EC30 values for the pollutant mixture and predator cue were selected based on those predicted to have negligible effects (EC10) and moderate effects (EC30) – whilst still remaining within environmentally relevant levels. Similarly, the EC30 level for temperature was based on a combination of predicted moderate effects alongside general global warming predictions (+ 2°C), for which, organisms inhabiting small water bodies are highly susceptible. We investigated effects across a wide range of endpoints that are central to population stability (survival, growth, reproduction) over a 4.5-month exposure duration. The aim of this work was to explore interactions between stressors at low and environmentally relevant levels and combinations for chronic stressor exposures. We hypothesised that lower overall stressor intensity would result in fewer effects on measured endpoints, compared to the control organisms.

2. Methods

2.1. Experimental design

The basic experimental setup consisted of five replicates per treatment with each replicate containing 10 *L. stagnalis* embryos and 1600 mL modified artificial freshwater (mAFW: prepared according to OECD guidelines (OECD, 2016), except calcium levels were increased from 80 mg/L to 100 mg/L to ensure sufficient calcium availability throughout the full life-cycle exposure as OECD test is a 28 day adult exposure). A laboratory stock of *L. stagnalis* was established using 50 adults (*c*. 15–20 mm length) originating from the Somerset Levels, England (kindly donated by S. Dalesman, Aberystwyth University) combined with supplemental snails purchased in 2021 (Blades Biologicals Ltd, UK). Snails were housed in 10 L aerated aquaria and maintained in water baths (Grant Instruments, Sub Aqua Pro18) at 20 °C with 16:8 light-dark cycle. Water changes (50 %) were carried out twice per week, including the removal of faeces, and snails were fed organic lettuce *ad libitum* supplemented with fishflakes (Tetramin) once *per* week. Single embryos were separated from egg masses and were individually placed in exposure beakers (mAFW: 1600 mL). For further details see Moore et al. (2021). Water changes and experimental observations were conducted three times per week for 122 days post-hatching (see Supplemental Figure S1). The endpoints measured during the experiment were: total eggs produced (expressed as total viable eggs), onset of egg laying (in days after hatching), shell length (in mm), snail mass (in g), time to hatch, survival, avoidance behaviour (proportion of snails in the beaker that were above the waterline in the first hour after cue addition/water change). To assess the inter-experimental reliability, we compared survival in response to the different treatments observed in this experiment to that reported in Moore et al. (2021).

Table 1
Calculated exposure levels for each stressor (see Supplemental Table S1 for concentrations of each component within the pollutant mixture at the respective exposure levels).

Stressor	EC10	EC30
Pollutant mixture (Poll)	0.015 μg/L	0.041 μg/L
Invasive predator cue (Pred)	10 % cue	57.5 % cue
Temperature (Temp)	-	22 °C

2.2. Exposures

Exposure levels were selected based on the dose-response relationship on survival from single stressor experiments The pollutant mixture was originally derived by using publically available data for pollutant levels in European FWs, with components either dissolved in mAFW or acetone (for more details see Moore et al., 2021 & Table S1). The predator cue was derived by feeding *Pacifastacus leniusculus* with *Lymnaea stangalis*, and then one hour later, collecting/straining the water, before adding to exposure tanks. For the pollutant mixture and the predator cue, two stressor levels were selected: EC10 – i.e., the level of each stressor eliciting a 10 % effect compared to the control; and EC30 – the level of each stressor eliciting a 30 % effect compared to the control (see Supplemental Figure S2). Additionally, temperature was included as the third stressor, at the approximated EC30 level (this could not be computed precisely due to the apparent biphasic shape of some of the stressor-response curve, see Supplemental Table S1 & Supplemental Figure S2). This resulted in a total of 13 experimental conditions (2 x controls [control & solvent control]; 5 x single stressor; 3 x binary stressor; 3 x ternary stressor, Table 2). We did not use a fully factorial design due to space limitations, and stressor combinations and levels were selected based on scientific interest.

No chemical analysis was carried out on the pollutant mixture, because the target contaminant concentrations in the exposure tanks were below the detection limits of the available testing laboratories (see Supplemental Table S1 for nominal pollutant levels). Stock solutions and spiking of the exposure medium was prepared as previously described (Moore et al., 2021). To determine levels in new stock solutions and for any uptake of chemicals, stocks and snail soft tissue was collected upon termination of the experiment and sent for analysis, however, these samples were unfortunately lost in transit. However, chemical analysis was carried out for the previous study which used the same experimental design (Moore et al., 2021) with levels within 10 % of the nominal

Table 2
Treatments tested in this experiment.

Treatment Name	Treatment	No. stressors	Poll. 0.015 μg/L	Pred. 10 % cue	Poll. 0.041 μg/L	Pred. 57.5 % cue	Temp. 22 °C	Solvent
Control	Control	0						N
Solvent control	Solvent control (0.0005 % acetone)	0						Y
PollEC30	Pollutant mixture, EC30	1			Y			Y
PredEC30	Predator cue, EC30	1				Y		N
TempEC30	Temperature, EC30	1					Y	N
PollEC10	Pollutant mixture, EC10	1	Y					Y
PredEC10	Predator cue, EC10	1		Y				N
PollPredBinary	PollEC10 +PredEC10	2	Y	Y				Y
PredBinary	PollEC10 +PredEC30	2	Y			Y		Y
PollBinary	PollEC30 +PredEC10	2		Y	Y			Y
TempTernary	PollEC10 +PredEC10 +TempEC30	3	Y	Y			Y	Y
PollTempTernary	PollEC30 +PredEC10 +TempEC30	3		Y	Y		Y	Y
PollPredTempTernary	PollEC30 +PredEC30 +TempEC30	3		Y		Y	Y	Y

concentrations (Table S2).

2.3. Statistics

To evaluate the influence of the treatments on the different responses that were measured in the experiment, we ran multiple statistical models and also considered potential interactions between treatment variables. Prior to analysis, a single response variable was chosen among groups of highly correlated variables (Pearson correlation coefficient $|r| > 0.6$ with $p < 0.05$). This resulted in the exclusion of shell width and length, because both were strongly correlated with weight (width: $r = 0.851$, $p < 0.001$; length: 0.861, $p < 0.001$).

We used generalized linear models (GLMs) to investigate treatment effects on those endpoints with a single measurement per beaker (time until hatching, onset of egg production, number of viable eggs, survival rate). Temperature (T), predator cue (PC) and pollutant mixture (PM) were included as predictors, as well as the two-way interaction between T and PM, and between PM and PC:

$$link(Endpoint) = T + PC + PM + T \times PM + PM \times PC \quad (1)$$

The experimental design lacked a few treatment combinations. As a consequence, regression models including the three-way interaction, or

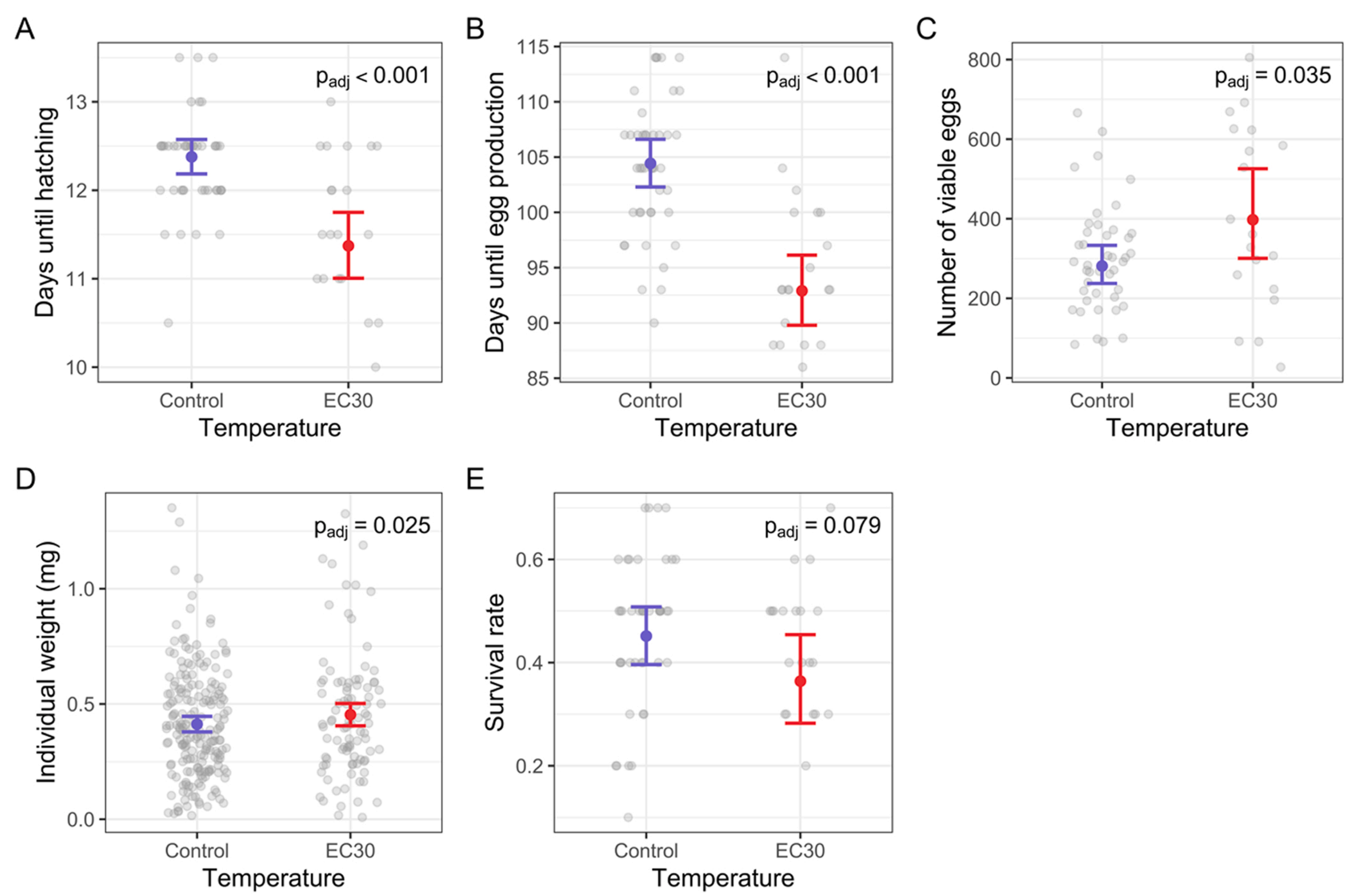

Fig. 1. Temperature effects on measured endpoints (control = 20 °C, EC30 = 22 °C). Marginal regression model predictions are plotted along with their 95 % confidence interval. Grey points represent the raw data ($N_{control} = 43$, $N_{EC30} = 20$ for A-C and E, $N_{control} = 195$, $N_{EC30} = 94$ for D) and adjusted FDR-p-values for ANOVA type II tests from the regression models are indicated in the upper right corner.

including the interaction between T and PC in addition to another two-way interaction were rank-deficient. Therefore, we fitted only the interactions between T and PM, as well as between PM and PC. The distributional family for the GLM was chosen according to the response variable (gamma GLM with log link for time until first hatching and onset of egg production; negative binomial GLM with log link for number of viable eggs; binomial GLM with logistic link for survival rate) and model assumptions were graphically evaluated with regression plots using the package DHARMa (version 0.4.6,Hartig, 2024; see Supplemental Text S1).

For the response variables avoidance behaviour and individual weight, the data consisted of multiple measurements per beaker. To account for multiple measurements, we fitted generalized linear mixed effects models (GLMMs; proportion of snails above the water line: binomial GLMM with logistic link, individual weight: gaussian GLMM with identity link) with the same combination of explanatory variables, and the beaker as random effect. Avoidance behaviour was measured at different days and we included the day of the experiment as a second random effect.

In addition, we explored if avoidance behaviour (AV) ameliorated the effect of stressors on the other response variables. For this purpose, we fitted GLMs as indicated above, however, with a modified regression equation:

$$link(Endpoint) = T + PC + PM + AV + T \times AV + PC \times AV + PM \times AV \quad (2)$$

AV was calculated per beaker as the average proportion of snails above the water line across observations and the analysed endpoints were onset of egg production, number of viable eggs, survival rate and average snail weight within a beaker.

P-values were adjusted based on the false discovery rate (FDR) (Benjamini and Hochberg, 1995) within the original set of GLMs and within the GLMs of the exploratory analysis, respectively. Adjusted p-values of < 0.001 were deemed as well-supported effects, and values in the range p 0.001–0.1 as potential effects, where the null hypothesis could not be definitively rejected (Wasserstein, Schirm and Lazar, 2019). Effect sizes were calculated as response ratios (RR) relative to control conditions, if not indicated otherwise, using the marginal effects package (version 0.22.0, Arel-bundock et al., 2024) and are reported along with their standard error.

3. Results

3.1. Water quality

Water quality parameters were close to target conditions (20 °C, pH 7.5–8.5, 650–850 µS/cm, > 60 % DO: OECD, 2016) across the experimental period for all treatments and there were no differences between treatment groups other than for the intentional experimental exposure (i.e., temperature; see Supplemental Table S3).

3.2. Experimental reliability

The control and solvent control were similar for all of the measured endpoints. In the single stressor exposures, the survival rates expected from our previous experiment for all single stressor treatments was within 10 %, except for one replicate beaker in the Pollution EC10 treatment, which was omitted from analysis due to 100 % mortality early in the experiment (at 44 days post-hatch [dph]; see Supplemental Table S4). Although not directly comparable – due to differing exposure levels – generalised responses to single stressors in this experiment were similar compared to those reported in Moore et al. (2021); stimulated reproduction in response to elevated temperature, increased avoidance behaviour in response to the predator cue and a tendancy for fewer viable eggs in response to the pollutant mixture). See Supplemental Figure S3.

Table 3
ANOVA table for main analysis. Type II ANOVA was conducted for each fitted regression model to obtain the interaction and main effects of the predictors temperature (T), pollutant mixture (PM), and predator cues (PC). P-values were FDR-adjusted for each predictor.

Endpoint	Predictors	χ^2	df	Effect Sign	FDR-adjusted p value
Time until hatching	T	18.7	1	+	< 0.001
	PM	1	2		0.973
	PC	3.5	2		0.26
	T x PM	5.4	2		0.328
	PM x PC	9	4		0.183
Onset of egg production	T	34.1	1	+	< 0.001
	PM	3.1	2		0.636
	PC	2.6	2		0.323
	T x PM	0.9	2		0.636
	PM x PC	10.1	4		0.183
Number of viable eggs	T	5.1	1	+	0.035
	PM	0.3	2		0.973
	PC	8.6	2	-	0.082
	T x PM	3.4	2		0.328
	PM x PC	2.3	4		0.675
Snail weight	T	6.2	1	+	0.025
	PM	3.2	2		0.636
	PC	0	2		0.987
	T x PM	3	2		0.328
	PM x PC	6.7	4		0.309
Avoidance behavior (Proportion of snails above water line)	T	0.2	1		0.638
	PM	0.1	2		0.973
	PC	4.8	2		0.185
	T x PM	2.6	2		0.328
	PM x PC	3.1	4		0.643
Survival rate	T	3.4	1	-	0.079
	PM	0.1	2		0.973
	PC	5.1	2		0.185
	T x PM	3.1	2		0.328
	PM x PC	5.4	4		0.375

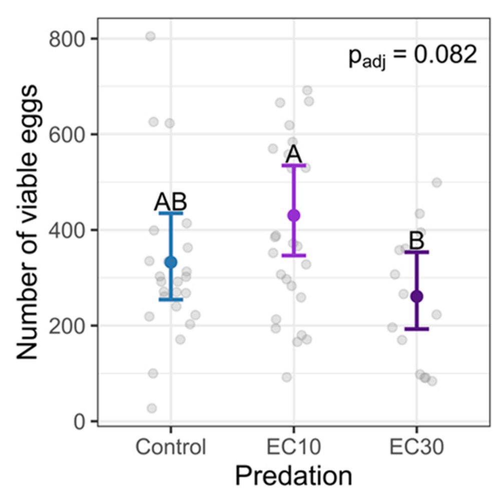

Fig. 2. Predation effect on the number of viable eggs (EC10 = 10 % predator cue; EC30 = 57.5 % predator cue). Marginal regression model predictions are plotted along with their 95 % confidence interval. Grey points represent the raw data ($N_{control} = 24$, $N_{EC10} = 25$, $N_{EC30} = 14$). The group labels (A and B) indicate which group differences were supported in post-hoc tests.

Single and Combined Effects of Elevated Temperature

Elevated temperature resulted in an acceleration of the time until hatching ($\chi^2_1 = 18.7$, $p_{adj} < 0.001$, RR $= 0.925 \pm 0.018$; Fig. 1A) and timing of the onset of egg laying ($\chi^2_1 = 34.1$, $p_{adj} < 0.001$, RR $= 0.888 \pm 0.018$; Fig. 1B). In addition, elevated temperature increased the number of viable eggs ($\chi^2_1 = 5.1$, $p_{adj} = 0.035$, RR $= 1.483 \pm 0.253$; Fig. 1C) and individual weight of the snails ($\chi^2_1 = 6.2$, $p_{adj} = 0.025$, RR =

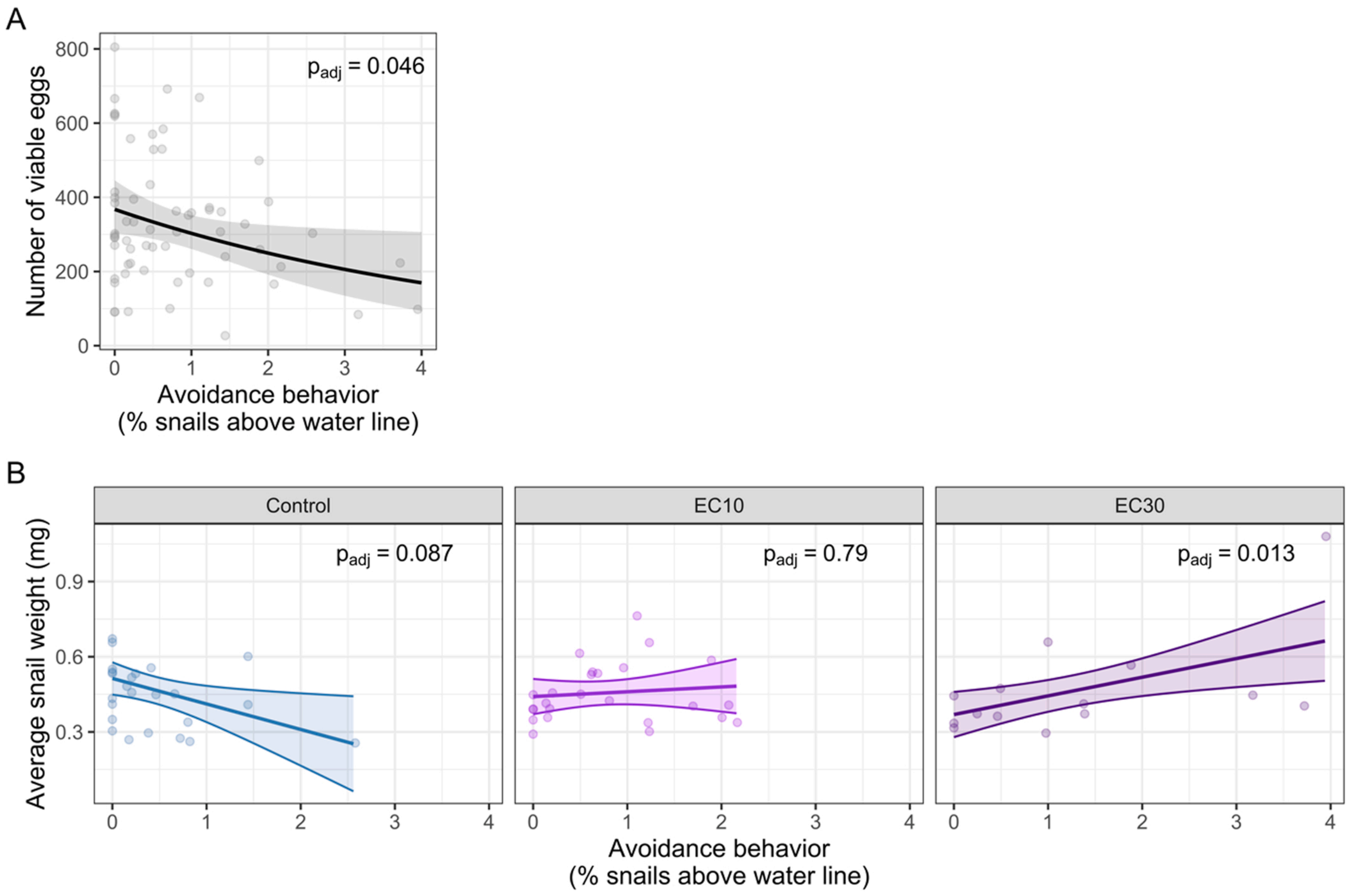

Fig. 3. Relationships between avoidance behavior and (A) the number of viable eggs (N = 63) and (B) average snail weight dependent on the predation treatment ($N_{control}$ = 24, N_{EC10} = 25, N_{EC30} = 14). EC10 = 10 % predator cue; EC30 = 57.5 % predator cue. Regression lines indicate the marginal predictions of the regression models and the shaded area indicates the 95 % confidence interval. FDR-adjusted p-values are given in the upper right corner of each plot.

1.247 ± 0.107; Fig. 1D). Negative impacts of elevated temperature in terms of a roughly 20 % reduction in survival were also observed, though not statistically significant when adjusted for multiple testing (χ^2_1 = 3.4, p_{adj} = 0.079, RR = 0.805 ± 0.102, Fig. 1E). No additional effects were observed for temperature treatments when combined alone or together with the pollutant mixture and predator cue (Table 3).

Single and combined effects of pollutant mixture & predator cue

Negative impacts were observed for the addition of predator cues on the number of viable eggs, but again not statistically significant when adjusted for multiple testing ($\chi^2_2 = 8.6$, $p_{adj} = 0.082$, Fig. 2). In post-hoc comparisons, differences were only detected between the EC30 and EC10 predation treatments (RR EC30/EC10: 0.600 ± 0.111, p_{adj}<0.001) and the number of viable eggs was lower in the EC30 predation treatment. Neither pollutant mixture nor predator cues had other significant effects on any of the endpoints, either singly or combined (Table 3).

Behavioural Responses

Higher avoidance behaviour was correlated with a reduced number of viable eggs when pooled across all treatment groups ($\chi^2_1 = 6.1$, p_{adj} = 0.046, RR = 0.862 ± 0.086, Fig. 3A). Spending time above the water line was also associated with the average weight of snails within a beaker ($\chi^2_1 = 5.2$, $p_{adj} = 0.046$). However, the direction of this effect depended on the predation treatment (interaction between avoidance behavior and predation: $\chi^2_2 = 14.6$, $p_{adj} = 0.003$). If no predator cues were added, average snail weight did not differ there statistically significantly (negative trend: $p = 0.087$; Fig. 3B left).By contrast, in predation treatments snail weight was associated with avoidance behaviour (positive trend: $p = 0.013$; Fig. 3B right). See Table 4 for list of statistical groups and associated p values.

4. Discussion

We investigated the combined impact of a pollutant mixture, invasive predator cue and elevated temperature on a range of life-history endpoints using a partial-lifecycle experimental design. Effects were observed on all measured endpoints in response to at least one treatment, with the most pronounced effects observed in response to elevated temperature. Survival in the single-stressor exposures was consistent with our previous experiment for each stressor alone, indicating good reproducibility between experiments. The solvent had no effect on any endpoint, which is in agreement with earlier experiments (Bandow and Weltje, 2012; Moore et al., 2021). Further, the responses of the snails displayed similar trends compared to those we previously reported (Moore et al., 2021) and the single stressor responses at the EC30 level reported here.

Temperature elicited the most pronounced effects across endpoints. This implies stimulation of physiological processes, evidenced by accelerated developmental, reproduction and growth rates, compared to controls – although, interestingly, this may not in fact be due to enhanced metabolic rate (Hahn, 2005). Although the associated mechanisms for this stimulation remain unclear, a similar response has been reported in *Daphnia pulex*, whereby population growth rate was overwhelmingly dominated by elevated temperature, with the addition of pyrimethanil or predator kairomones eliciting relatively minor effects (Scherer et al., 2013). Despite survival being reduced by elevated temperature (20 % lower, not signficant, $p = 0.079$), the total reproductive output *per* beaker was higher (48 % higher). Therefore, on average, each surviving snail produced ~66 % more viable eggs than control snails (80 % of the snails produce 133 % of the eggs, therefore 133 % per 80 %

Table 4
ANOVA table for exploratory analysis. Type II ANOVA was conducted for each fitted regression model to obtain the interaction and main effects of the predictors avoidance behaviour (AV), temperature (T), pollutant mixture (PM), and predator cues (PC). P-values were FDR-adjusted for each predictor.

Endpoint	Predictors	χ^2	df	Effect Sign	FDR-adjusted p value
Onset of egg production	T	23.7	1	+	< 0.001
	AV	1.9	1		0.23
	PM	2.4	2		0.591
	PC	0.9	2		0.728
	T x AV	1	1		0.626
	PM x AV	4.5	2		0.143
	PC x AV	0.4	2		0.828
Number of viable eggs	T	2.1	1		0.192
	AV	6.1	1	-	0.046
	PM	0.2	2		0.899
	PC	5	2		0.327
	T x AV	3.6	1		0.23
	PM x AV	5.3	2		0.142
	PC x AV	0.9	2		0.828
Survival rate	T	0.6	1		0.452
	AV	0.2	1		0.675
	PM	0.4	2		0.899
	PC	1.1	2		0.728
	T x AV	0.2	1		0.863
	PM x AV	1.3	2		0.535
	PC x AV	0.7	2		0.828
Snail weight	T	2.8	1		0.188
	AV	5.2	1	-	0.046
	PM	2.5	2		0.591
	PC	0.6	2		0.728
	T x AV	0	1		0.889
	PM x AV	5.3	2		0.142
	PC x AV	14.6	2	+	0.003

= 166 %). This supports a well-known theory for a trade-off between survival and reproduction – whereby organisms at higher probability of mortality by extrinsic factors allocate more resources to reproduction – which has been demonstrated experimentally in various trematode parasite species (Hechinger, 2010). This response (lower survival, higher reproduction) was also reported in our previous experiment using *Lymnaea stagnalis* (Moore et al., 2021). Further, two studies have reported higher reproduction in response to heatwave events in *Lymnaea stagnalis*, both employing a short-term/adult only experimental design (32 days: Salo et al., 2017; 11 days: Leicht et al., 2013). In both studies, no impact on mortality was observed – perhaps due to the short time-frame of the exposures – however, in the latter study the authors hypothesised the positive effects on reproduction may have been due to a perceived threat to survival. In this study, we did not measure offspring fitness, and therefore it remains open how these seemingly positive population level impacts on reproductive output might translate to population recruitment. However, some evidence suggests that maternal heatwave results in offspring with decreased fitness in *Lymnaea stagnalis* (Leicht and Seppälä, 2019). We are not aware of long-term studies in molluscs (or other aquatic animals) where a range of physiological endpoints have been investigated using a partial life-cycle design to compare our responses.

Avoidance behaviour was not impacted by elevated temperature, which was suprising given that temperature extremes are commonly found in small, shallow water bodies, and avoidance behaviour is a strategy employed by air-breathing snails (i.e., gastropods)., However, as expected, predator cues induced avoidance behaviour. Exploratory analyses revealed that snails exposed to predator cues benefitted from spending more time above the water line by having a higher body mass than those spending more time submerged (statistically significant at the EC30 level). This trend was not observed for snails that were not exposed to predator cues, where spending more time above the water line tended to lead to a reduced body mass ($p = 0.087$). This response of unexposed snails was expected because while snails are above the water line, they are unable to simultaneously feed as the available food resource was located in the aquatic media. The mechanism by which snails can increase their body mass while not able to feed is unknown and intriguing. We are not aware of comparable studies – whereby correlative analyses have been used to assess relationships between avoidance behaviour and other endpoints, however, predator cue has previously been shown to result in reduced growth in a freshwater gastropod (*Potamopyrgus antipodarum,* avoidance behaviour was not investigated in that study: Levri et al., 2020). There may be trade-offs occurring, whereby avoiding the stressful perceived predator presence, potentially results in a higher surplus of energy resources available for growth; however, this is a hypothesis only in the absence of supporting data.

Surprisingly, there were no significant effects of the pollutant mixture on any endpoints. A meta-analysis on the effects of toxicants paired with just one other environmental stressor revealed that the presence of an additional stressor increased an organisms sensitivity to a toxicant up to 100 fold at an EC10 exposure level (Liess et al., 2016); however, our results strongly contrast with those. In this study, conversely, the addition of pollutant mixture (and the predator cue) tended to reduce the effects of temperature compared to the temperature EC30 alone. This was evident for reproductive endpoints (timing onset of egg laying [mean, days]: temperature EC30 [92.2] *v* EC30 +EC30 +EC30 [100.8] *vs.* control [106.4]; number of viable eggs [mean, number]: temperature EC30 [370.8] *v* EC30 +EC30 +EC30 [141.8] *vs.* control [213.2]) and for growth ([mean, grams] temperature EC30 [0.54] *v* EC30 +EC30 +EC30 [0.35] *vs.* control [0.36]). Although the majority of studies has reported that toxicants exacerbate the effects of temperature, an amelioration of effects of elevated temperature have been reported previously in several organisms: in *L. stagnalis,* the addition of a pollutant mixture neutralised the positive effect of temperature on reproduction (i.e., temperature increased reproduction, temperature + pollutant = control) upon a 32 day exposure of adults (Salo et al., 2017). In *Daphnia pulex* the positive effects of temperature on reproduction were neutralised upon exposure to pyrimethanil or predator kairomones for 35 days, (Scherer et al., 2013). Somewhat conversely, in deepwater corals, negative effects of higher temperature were observed on growth, but these negative effects were less pronounced upon co-exposure to lower pH and fewer food resource (6-month exposure, Beck et al., 2023). Although accelerated growth and stimulated reproduction can be considered as 'positive' effects, intriguingly, this pattern was also observed for survival, with higher survival observed in response to the multiple stressor exposure compared to elevated temperature alone ([mean, %], temperature EC30 [38] *v* EC30 +EC30 +EC30 [54] *vs.* control [57], which has not been previously reported. This is difficult to explain currently, however, warrants further investigation as this pattern has been observed in response to various stressors combined with elevated temperature in a range of organisms.

Overall, the findings from this study contradict the predictions of typical multiple stressor models suggesting additivity (sum of stressor effects), synergy (higher than expected effects) or dominance (effects observed in response to single stressor ~ multiple stressor), while some of the results match an effect reversal (two negative effects become positive). In conclusion, we report here for the first time the effects of environmentally relevant exposures to multiple stressors, over a long-time period and encompassing a wide range of apical endpoints that are central to population maintenance. In particular, the effects of elevated temperature, which are within current temperature increases (+2°C) – with small, shallow water bodies particularly at risk of temperature extremes – elicited profound effects on our test species, and these were more pronounced than for the pollutant mixture or the predator cue.

CRediT authorship contribution statement

Frances Orton: Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Data curation, Conceptualization. **Claus Svendsen:** Writing – review & editing, Conceptualization. **Ralf B. Schäfer:** Writing – review & editing, Formal analysis. **Iris Madge Pimentel:** Writing – review & editing, Formal analysis. **Emily M. Moore:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2025.119505.

Data availability

Data will be made available on request.

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