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A laboratory-based comparison for the effects of environmental stressors supports field evidence for the relative importance of pollution on life history and behaviour of the pond snail, *Lymnaea stagnalis*

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Pollution, temperature, climate change, invasive species, freshwater, Mollusca, multiple stressor, ecotoxicology

Synopsis

This research demonstrates the relative impacts of three ubiquitous stressors upon full-life cycle exposure and supports field-based evidence of stressor ranking which is important for environmental risk assessment.

Abstract

Biodiversity is declining at an alarming rate globally, with freshwater ecosystems particularly threatened. Field-based correlational studies have 'ranked' stressors according to their relative effects on freshwater biota, however, supporting cause-effect data from laboratory exposures are lacking. Here, we designed exposures to elicit chronic effects over equivalent exposure ranges for three ubiquitous stressors (temperature: 22-28 °C; pollution [14 component mixture]: 0.05-50 µg/L; invasive predator cue [signal crayfish, *Pacifasticus leniusculus*]: 25-100 % cue) and investigated effects on physiological endpoints in the pond snail (*Lymnaea stagnalis*). All stressors reduced post-hatch survival at their highest exposure levels, however, highly divergent effects were observed at lower test levels. Temperature stimulated hatching, growth and reproduction whereas pollution delayed hatching, decreased growth, reduced egg number/embryo viability and induced avoidance behaviour. The invasive predator cue stimulated growth and reduced embryo viability. In agreement with field-based ranking of stressors, pollution was identified as having the most severe effects in our test system. We demonstrate here the utility of laboratory studies to effectively determine hierarchy of stressors according to their likelihood of causing harm in the field, which has importance for conservation. Finally, we report negative impacts on life-history traits central to population stability (survival/reproduction) at the lowest pollution level tested (0.05 µg/L).

Introduction

There is a pressing need to understand the ways in which humans negatively impact biota in order to predict and reduce the rate of biodiversity loss[1]. Habitat loss is generally recognised as the most severe threat facing biota, but other environmental stressors - defined as factors that disrupt the performance, fitness or homeostasis of an organism, population or ecosystem[2]- also contribute to species declines. Freshwater (FW) ecosystems are amongst the most threatened globally[3] with environmental stressors such as pollution, climate change and non-native species invasions reported to negatively impact FW biota[reviewed in 4,5]. Multi-variate analyses of field collected data have recently

been employed to 'rank' environmental stressors according to their potential for harm in FW ecosystems by analysing for correlations between stressor intensity and biodiversity levels. It has been reported that pollution, in the form of eutrophication, may be one of the most detrimental to FW taxa compared with other stressors[6,7], and habitat alterations (e.g. drainage, flow, cover, water chemistry[8,9]) also rank amongst the biggest drivers of biodiversity loss in freshwater systems, which potentially may be driven by climate change[e.g. 10,11].

The purpose for the ranking of environmental stressors in the field environment is to design effective mitigation strategies for the protection of FW biodiversity. However, without supporting laboratory derived data to underpin these findings with cause-effect data, correlational analyses lack reliability and robustness. To date, ranking of environmental stressors in laboratory studies has been undertaken using modelling to identify the relative effects of stressors when applied together in a multiple stressor experimental design[e.g. 12,13]. However, this approach does not allow investigation into the individual toxicities of these stressors. Since stressors are present at almost infinite combinations of levels and identities, investigations into their potencies when applied singly to assess their relative potential impacts on biota are also needed.

For this study, the stressors we selected for testing were sub-optimal temperature, pollution and invasive predator cue as these are amongst the most ubiquitous stressors impacting on FW ecosystems[14]. For temperature, sub-optimal levels were characterised by higher mean temperature exposure, as the mean global freshwater temperature is predicted to rise as a result of climate change[15]. For the pollution stressor, chemical contaminants have been detected in virtually all FW environments[16] and specifically chemical mixtures have importance since chemical pollutants are virtually never found singly in environmental samples[14,15]. Likewise, invasive species are widespread globally, particularly in the Western Hemisphere and newly industrialised countries (e.g. China, Brazil, India) where trade and transport are common practice[19]. Furthermore, each of these stressors

75 are known to have negative impacts on FW invertebrates (temperature[reviewed in 20]; pollutants[reviewed in
76 21]; invasive species[reviewed in 22]) which make up 60 % of named freshwater taxa[23].

77 FW molluscs are the most diverse FW taxa yet they are also the most highly threatened FW animal
78 group – with an estimated 50 % of species threatened compared to 37 % of fish and 23 % of
79 amphibians[24], and therefore, an important group to test the effects of environmental stressors. In
80 this study, we investigated the effects of higher temperature, a pollutant mixture and invasive
81 species predator cue on hatching, survival, growth, reproduction and avoidance behaviour in the
82 great pond snail (*Lymnaea stagnalis*). As well as belonging to a highly threatened FW Phylum, *L.*
83 *stagnalis* is commonly utilised as a model species for testing the effects of stressors[e.g. 25], and is used
84 as an Organisation for Economic Co-operation and Development (OECD) test species for identifying
85 endocrine disruptors (28 day adult exposure[26]). Importantly, we also exposed *L. stagnalis* across
86 their full life-cycle which is more environmentally relevant than short-term exposures. To the
87 authors' knowledge, full life-cycle exposure to neither temperature, a pollutant mixture containing
88 more than three components nor invasive predator cue in any mollusc species has been reported to
89 date. However, we hypothesise from published literature on shorter term exposures that the
90 pollutant mixture will have negative impacts on hatch success[27], temperature will stimulate growth
91 and thus onset of reproduction[28,29] and the invasive species cue will stimulate avoidance
92 behaviour[30]. The overall aim of this study was to compare and rank stressors for their effects on life
93 history traits in *L. stagnalis*. Thus, exposure levels of each stressor were scaled with the aim of
94 eliciting a wide range of chronic effects for each stressor in order to facilitate analysis of the
95 comparative effects.

Methods

Lymnaea stagnalis Husbandry

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). Snail stocks were maintained in 10 L aquaria filled with modified artificial freshwater (mAFW). This was prepared according to OECD guidelines (ISO 6341:2012[26]), except calcium levels were increased from 80 mg/L to 100 mg/L to ensure sufficient calcium availability throughout the full life-cycle exposure (OECD test is a 28 day adult exposure). Aquaria were aerated and maintained in waterbaths (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.

Experimental Design

For each exposure experiment (temperature, pollution and invasive predator), eight snail egg masses were removed from stock tanks (< 24 hours old, > 30 embryos *per* mass) for use in the experiment. To reduce potential impact of parental origin, single embryos were separated from egg masses and were individually placed in 2 L beakers containing aerated mAFW (1600 mL), with each beaker containing one embryo from each of the egg masses (n = 8) and five replicate beakers *per* experimental condition (n = 25 beakers *per* experiment). Hatching typically occurs between day 12-14 under control conditions[31], therefore a hatching period of 21 days was designated to allow for delayed hatching as a result of stressor exposure; unhatched embryos after this time were deemed non-viable. Water changes were carried out once per week prior to hatching and three times per week thereafter (Monday, Wednesday, Friday) until 122 days (~ 4 months) post-hatch. Prior to water changes, water quality parameters (temperature, pH, dissolved oxygen, conductivity, nitrate/nitrite) and mortality were recorded in each beaker (any dead snails were removed). Immediately following each water change, avoidance behaviour (the number of snails above the waterline) was recorded every five minutes for 1 hour beginning on day 20 post-hatch. For each experiment, day zero post-hatch was determined as the median day at which 50 % of embryos had hatched across all exposure

levels and was used to standardise sampling day across all conditions within experiments. Once snails reached reproductive maturity (median = 92 days post-hatch across all experiments), reproductive output (fecundity, embryo viability) was monitored weekly until 122 days post-hatch. Embryo viability was calculated as the percentage of embryos with a normal appearance (single developing embryo within the egg casing) and fecundity (total egg number) was calculated from the cumulative number of eggs produced in each beaker by the end of the experiment. The number of surviving snails was counted at the end of the experiment (day 122 post-hatch) to determine the survival percentage of snails that remained from the total hatched.

Sampling Procedure

Snail mass and shell length were recorded on days 60, 80, 100 and 122 post-hatch. To measure snail mass, excess water on the shell was blotted with tissue paper prior to placing on a scale. Where snail mass was below the calibration limit of the scales, which occurred on day 60 only, mass was recorded as 0.001 g (percentage of snails below calibration limit for each exposure: temperature = 0 %, pollutant mixture = 8.0 %, invasive predator cue = 1.9 %). Shell length was measured from the front of the aperture to the apex of the shell using digital callipers (see supplemental Fig. S1). See supplemental Figure S2 for graphical depiction of experimental design and sampling timeline.

Exposures

The highest exposure level for each stressor was selected based on prior knowledge of medium-high effect levels for *L. stagnalis* (see supplemental Table S1) and three further levels were then selected by scaling down from this "high" level, with each exposure also containing a control condition (temperature: 20 [control] 22, 24, 26 and 28 °C, pollutant mixture: 0 [control] 0.05 , 0.5, 5 and 50 µg/L; invasive predator cue: 0 [control] 25, 50, 75 and 100 %). Additionally, a solvent control (0.0005 % acetone) was compared against a mAFW control to confirm the previously reported lack of effect of acetone at this level in *L. stagnalis*[31]. The pollutant mixture and invasive predator cue exposures were conducted at 20 °C ("control" temperature).

Pollutant Mixture

The pollutant mixture comprised 14 components with high detection frequency in UK and European freshwaters (see supplemental Table S2). All components were purchased from Sigma Aldrich (Poole, UK): copper sulphate pentahydrate ("copper", CAS 7758-99-8), nickel chloride hexahydrate ("nickel", CAS 7791-20-0), propranolol (CAS 4199-10-4), metaldehyde (CAS 7791-20-0), benzotriazole (CAS 95-14-7), polychlorinated biphenyl 153 ("PCB153", CAS 35065-27-1), carbamazepine (CAS 298-46-4), ibuprofen (CAS 15687-27-1), triclosan (CAS 3380-34-5), bisphenol-A ("BPA", CAS 80-05-7), isoproturon (CAS 34123-59-6), pentadecafluorooctanoic acid ("PFOA", CAS 335-67-1), 2,4-dinitrophenol ("2,4-DNP", CAS 51-28-5) and 17α-ethinylestradiol ("EE2", CAS 57-63-6). Mixture components were applied at levels proportional to their median detected levels in European freshwaters, except for copper and nickel which were applied at x4 lower than the environmental median to reflect the comparatively high proportion of metals that bind to organics in natural systems[32] (see supplemental Table S2).

Stock solutions were prepared in acetone (10 combined components: benzotriazole, PCB153, carbamazepine, ibuprofen, triclosan, BPA, isoproturon, PFOA, 2,4-DNP, EE2) or mAFW (individual components: copper, nickel, propranolol, metaldehyde). The stock solutions were diluted to reach target concentration in the exposure water (x 200,000 dilution of acetone mixture stock, x 80,000 dilution of metaldehyde and propranolol stocks and x 8000 dilution of copper and nickel stocks). Serial dilutions of the 50 µg/L stock were used to prepare stocks for the lower exposure conditions so that each condition had an equivalent acetone volume (0.0005 % acetone). Nominal levels of individual components in the mixtures ranged from 0.83 ng/L (EE2) to 5.38 µg/L (copper) in the 50 µg/L exposure condition, with proportionally lower levels in other exposure concentrations (Table 1).

Table 1: Nominal concentrations (µg/L) for each component in the stock solutions and 50, 5, 0.5 and 0.05 µg/L exposure medium.

Component	Diluent	50 µg/L Stock	50 µg/L Exposure	5 µg/L Exposure	0.5 µg/L Exposure	0.05 µg/L Exposure
Copper	mAFW	43000	5.38	0.538	0.0538	0.00538
Nickel	mAFW	42500	5.23	0.523	0.0523	0.00523
Benzotriazole	Acetone mixture	575200	2.88	0.288	0.029	0.0029
PCB 153	Acetone mixture	285055	1.43	0.143	0.0143	0.00143
Carbamazepine	Acetone mixture	190885	0.95	0.095	0.01	0.001
Ibuprofen	Acetone mixture	117060	0.59	0.059	0.006	0.0006
Triclosan	Acetone mixture	114530	0.58	0.058	0.006	0.0006
Propranolol	mAFW	25400	0.32	0.032	0.003	0.0003
BPA	Acetone mixture	81440	0.41	0.041	0.004	0.0004
Metaldehyde	mAFW	25400	0.32	0.032	0.0032	0.0003
Isoproturon	Acetone mixture	10181	0.05	0.005	0.0005	0.00005
PFOA	Acetone mixture	9100	0.03	0.003	0.0003	0.00003
2,4-DNP	Acetone mixture	2545	0.01	0.001	0.0001	0.00001
EE2	Acetone mixture	165	0.00083	0.000083	0.0000083	0.00000083

Analytical Chemistry

Levels of all chemicals except for EE2 (below limit of detection) and PCB153 (not possible within the remit of this study) were analysed in stock solutions and in mAFW medium both before and after the water change on day 70 for 50, 5 and 0.5 µg/L exposure conditions (levels in the 0.05 µg/L were below detection limits). Metal compounds (copper and nickel) were measured using inductively coupled plasma mass spectrometry (stocks: Laboratorio Analítico Bioclínico, Spain; exposure media: UK Centre for Ecology and Hydrology [measured elsewhere due to LOQ]) and the remaining compounds (benzotriazole, carbamazepine, ibuprofen, triclosan, BPA, propranolol, metaldehyde, isoproturon, PFOA, 2,4-DNP) were measured using ultra performance liquid chromatography mass spectrometry (Laboratorio Analítico Bioclínico, Spain). See supplemental text S1 for further details of chemical analysis.

Invasive Predator Cue

Signal crayfish (*Pacifasticus leniusculus*) of a similar size (30-40 mm carapace length) were collected from New Galloway, Southern Scotland (Scottish Natural Heritage licence No. 143712). Crayfish (n = 4) were individually maintained in plastic tanks containing aerated 6 L mAFW, with gravel substrate and shelter (PVC pipe). *L. stagnalis* has been shown to adapt anti-predator responses depending on the presence of 'alarm cue' from conspecifics[33] and whether predators are feeding on conspecifics[34], therefore crayfish cue water (CCW) was prepared by feeding two randomly selected crayfish (Microsoft Excel) one crushed *L. stagnalis* (15-20 mm shell length) 30 minutes prior to collecting 5 L of water from each snail-fed crayfish tank. . Cue dilutions were prepared by first straining CCW through a metal sieve lined with 1.2 mm plastic mesh (100% cue) and diluting with mAFW to produce 25, 50 and 75 % cue used for exposures. All crayfish tanks were emptied of any excess water, wiped down and replaced with fresh mAFW. Although predator cue concentrations were not measured these are known to decrease over time with prey responses upon exposure typically subsiding within 48 h[35], which was the minimum time period between water changes in this study (weekly over hatching period and Mondays, Wednesdays, Fridays post-hatch). There are currently no documented sightings of the invasive signal crayfish in the area that the *L. stagnalis* used for this study originated, but there have been confirmed observations of both invasive and the native (white-clawed crayfish, *Austropotamobius pallipes*) near to the Somerset Levels (2016-2020; NBN Atlas) as well as undocumented sightings of *P. leniusculus* after the time of snail collection. However, we cannot say for certain if our snail population has experienced *P. leniusculus* in the natural habitat.

Statistics

Data were analysed using R Software (version 3.6.1) in a series of Generalized Linear Mixed-Effects Models (GLMMs). For within experiment analyses, treatment groups were compared with their respective controls. "Exposure level" was considered as a fixed effect for within experiment analyses and "experiment" was considered as a fixed effect for between experiment analyses, with "beaker" included as a random factor in all analyses to account for replicate snails in the same beaker (glmer; R Package *lme4*[36]). Continuous data (hatching success, survival, growth, percentage of viable

embryos and water quality (temperature, pH, dissolved oxygen, conductivity)) were analysed using a Gamma distribution (with log link) whereas count data (time (days) to hatch, age (days post hatch) at onset of egg production and total eggs) were analysed using a Poisson distribution (with log link). Analysis of temperature between experiments did not include data where temperature was applied as a condition (i.e. data from 22-28 °C conditions were not included in between experiment analysis for temperature). Avoidance behaviour was modelled using a Generalised Additive Model in the R package *gamlss*[37] using "exposure level" as a fixed effect, "day" as a random factor to account for observations across time and with a zero-inflated beta distribution to account for a high proportion of zeros in the dataset. Pairwise comparisons (R package *emmeans*[38]) were applied to treatment levels within experiments for each endpoint (time to hatch, hatch success, survival, growth on 122 days post-hatch, onset of egg production, total eggs, embryo viability). To determine consistency between experimental controls and facilitate stressor ranking, pairwise comparisons were also applied between 'equivalent' exposure levels (i.e. 20 °C, 0 µg/L, 0%; 22 °C, 0.05 µg/L, 25 % ; 24 °C, 0.5 µg/L, 50%; 26 °C, 5 µg/L, 75 %; 28 °C, 50 µg/L, 100 %). Throughout, data are presented as mean ± 95% confidence intervals. *P* values of < 0.05 were deemed significant. See supplemental Table S5 for all test statistics.

Results

Experimental Conditions

Water quality parameters were optimal across the experimental period for all stressor experiments[26] and there were no significant differences in water quality parameters between condition groups or experiments (pH: 7.87 ± 0.4; dissolved oxygen: 83 ± 10%; conductivity: 740 ± 82 µS/cm ; temperature [except for where temperature was an exposure condition]: 20.05 ± 0.2°C). Nitrate remained <10 ppm across all experiments and conditions except for the 75 % and 100 % invasive predator cue conditions on day 53 post-hatch (10-20 ppm). As a result of this, an additional water change was carried out on day 54 to refresh test solutions and reduce nitrate levels which thereafter remained <

10 ppm. No effects of acetone were observed on any measured endpoints compared to mAFW controls (supplemental Fig. S1-S4).

Exposure levels

For the temperature stressor experiment, temperature levels remained close to nominal for each exposure condition (20 °C: 20.08 ± 0.21 °C; 22 °C: 22.01 ± 1.10 °C; 24 °C: 23.98 ± 0.21 °C; 26 °C: 25. 66 ± 0.22 °C; 28 °C: 27.95 ± 0.66 °C). All measured components in the pollutant mixture were detected in the stock solutions and measured levels of 11 of the 12 components were within 33 % deviation from nominal (copper, nickel, benzotriazole, carbamazepine, ibuprofen, propranolol, BPA, metaldehyde, isoproturon, PFOA and 2,4-DNP). Measured levels of triclosan deviated more widely (162.1 % of nominal) (Table 2). In the exposure media, broadly similar deviations from nominal were observed except triclosan deviated from nominal less in exposure medium compared to in the stock (79.8-119.7 % of nominal after the water change), however measured levels of copper and metaldehyde deviated from nominal more in exposure media (copper: 21.7 % of nominal; metaldehyde: 166.9-198.4 % of nominal) compared to in the stock solutions (copper stock: 133.0 % and metaldehyde stock: 132.6 % of nominal). Levels of components were typically higher after the water change compared to before the water change (Table 2).

255 Table 2: Nominal concentration of each component, level of quantification (LOQ), measured concentrations in 50 µg/L stock solutions (µg/L) and exposure medium before and after water

256 change (µg/L) with percentage of nominal concentration achieved (%). *CEH chemical analysis.

Component	**LOQ (µg/L)**	**50 µg/L Stock (µg/L)**			**50 µg/L Exposure Water (µg/L)**					**5 µg/L Exposure Water (µg/L)**					**0.5 µg/L Exposure Water (µg/L)**		
		Nominal	**Measured**	**%**	**Nominal**	**Before**	**%**	**After**	**%**	**Nominal**	**Before**	**%**	**After**	**%**	**Nominal**	**After**	**%**
Copper	10/0.02*	43000	57200	**133.0**	5.38	< LOQ		1.17*	**21.7**	0.538	<LOQ		<LOQ		0.0538	<LOQ	
Nickel	1/0.002*	42500	32913	**77.4**	5.23	5.00	**95.6**	5.01*	**95.8**	0.523	<LOQ		<LOQ		0.0523	<LOQ	
Benzotriazole	0.05	575200	694000	**120.7**	2.88	3.00	**104.3**	2.9	**100.8**	0.288	0.23	**80**	0.28	**97.4**	0.029	0.032	**113.4**
Carbamazepine	0.0008	190885	235400	**123.3**	0.95	1.60	**167.6**	1.6	**167.6**	0.095	0.13	**136.2**	0.14	**146.7**	0.01	0.013	**136.2**
Ibuprofen	0.5	117060	116500	**99.5**	0.59	<LOQ		<LOQ		0.059	<LOQ		<LOQ		0.006	<LOQ	
Triclosan	0.0008	114530	185600	**162.1**	0.58	0.56	**97.1**	0.69	**119.7**	0.058	0.022	**38.2**	0.046	**79.8**	0.006	0.0058	**100.6**
Propranolol	0.05	25400	20900	**82.3**	0.32	0.31	**97.6**	0.31	**97.6**	0.032	0.028	**88.1**	0.029	**91.3**	0.003	0.0034	**107**
BPA	0.1	81440	89900	**110.4**	0.41	0.24	**58.9**	0.25	**61.4**	0.041	<LOQ		<LOQ		0.004	<LOQ	
Metaldehyde	0.05	25400	33675	**132.6**	0.32	0.58	**182.7**	0.63	**198.4**	0.032	0.053	**166.9**	0.059	**185.8**	0.0032	< LOQ	
Isoproturon	0.0008	10181	9700	**95.3**	0.05	0.047	**92.3**	0.049	**96.2**	0.005	0.0034	**66.8**	0.0037	**72.7**	0.0005	< LOQ	
PFOA	0.0008	9100	9100	**100.0**	0.03	0.044	**115.2**	0.043	**112.6**	0.003	0.0035	**91.7**	0.0043	**112.6**	0.0003	0.0004	**105**
2,4-DNP	0.01	2545	2100	**82.5**	0.01	0.012	**94.3**	0.013	**102.2**	0.001	<LOQ		<LOQ		0.0001	<LOQ	

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Hatching and post-hatch survival

Time to hatch, overall hatching success and post-hatch survival were impacted by all of the stressors in response to at least one exposure level. Higher temperature stimulated hatching, with hatching occurring earlier in response to higher temperatures compared to the control (24 °C and 26 °C, $p < 0.003$; Fig. 1 A). Hatching success (the number of hatchlings) was reduced in response to higher temperatures (26 °C and 28 °C, $p < 0.02$) and the pollutant mixture (50 µg/L, $p = 0.01$) compared to their respective controls (Fig. 1 B). All the stressors negatively impacted post-hatch survival in response to at least one exposure level (22 °C and 28 °C: 0.05-50 µg/L: 25-100 %, $p < 0.04$) compared to their respective controls (Fig. 1 C). There were no differences observed between experiment controls for hatching success/time nor post-hatching survival. At medium (24 °C, 0.5 µg/L, 50 %) to high (28 °C, 50 µg/L, 100 %) exposure levels however, differences between stressors were observed with reduced hatch success in the temperature and pollutant mixture experiments compared to the invasive predator cue and lower survival in the pollutant mixture experiment compared with both the temperature and invasive predator cue experiments (Table S6).

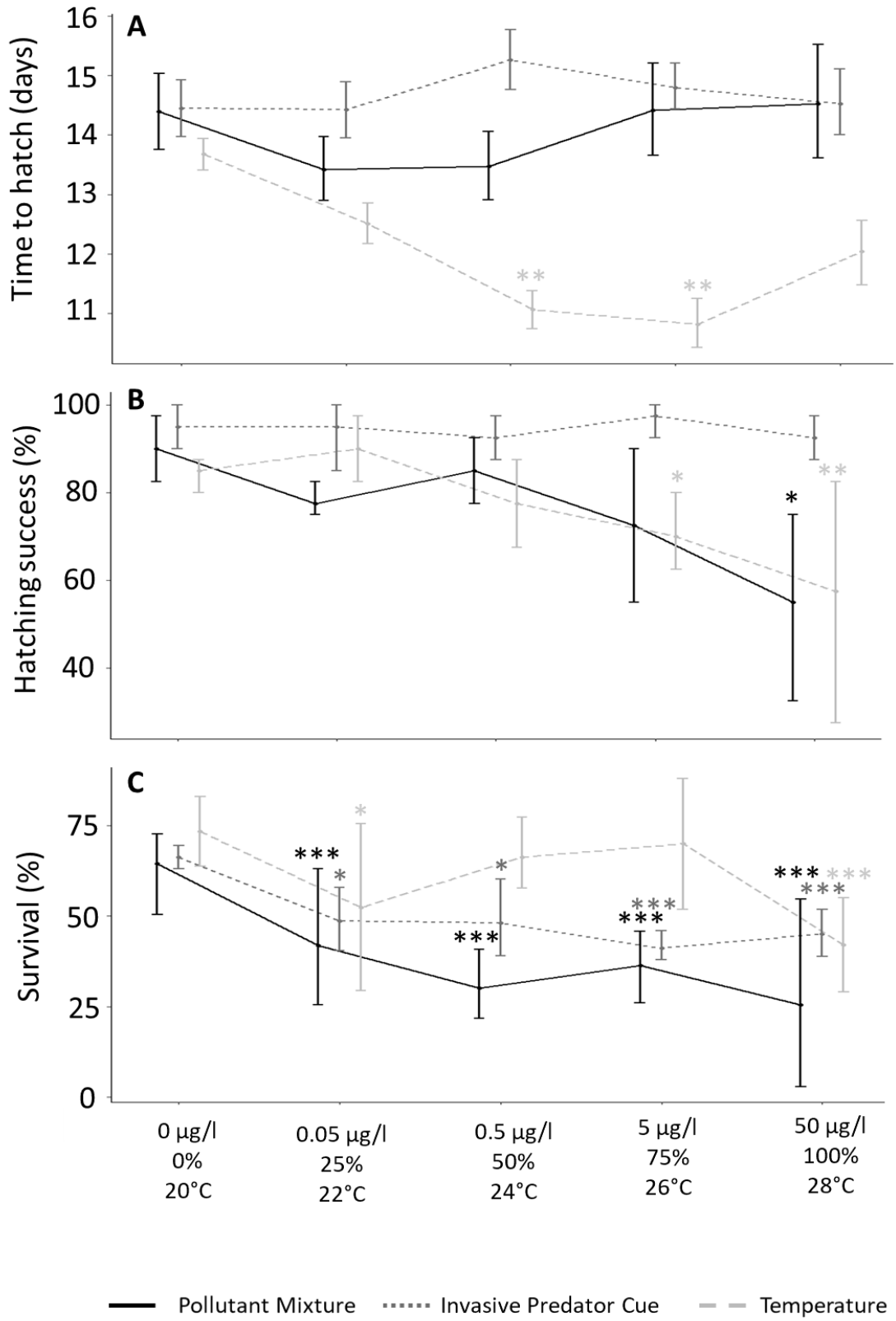


Figure 1: Mean ± 95 % confidence interval for (A) time to hatch in days, (B) hatching success after 21 days and (C) post-hatch survival (%) on day 122 across exposure levels for each stressor. See table S4 for *N* numbers for (A) and (C); (B) n=40 per exposure level per stressor. Significance to respective control is denoted by asterisks ('***'$p < 0.001$; '**' $0.001 < p < 0.01$; '*' $0.01 < p < 0.05$).

Growth

Compared to their respective controls, snail size (mass and shell length) was larger in response to higher temperatures (22-28 °C) and invasive predator cue (50-100 %) at all sampling time points (60, 80, 100, 122 days post-hatch, $p < 0.03$; Fig. 2 A-F). Exposure to the pollutant mixture had less uniform effects, with both larger and smaller sizes observed at different sampling days compared to the control (larger on days 60 and 80 in 5 µg/L condition, $p < 0.03$; Fig. 2 A, B, E, F; and smaller on days 80 and 100 in 50 µg/L condition, $p < 0.04$; Fig. 2 B, C). Differences in snail size were observed between stressor controls with snails in the invasive predator cue stressor experiment being smaller compared to both the pollutant mixture and temperature controls on days 80, 100 and 122 ($p < 0.001$; Fig. 2 B,F,G,H), whereas snails in the temperature stressor experiment were larger compared to both the invasive predator cue and pollutant mixture control snails on days 60 and 80 ($p < 0.001$; Fig. 2 A,B,E; Table S6). By the end of the experimental period snails in the invasive predator cue experiment were smaller compared with those in the temperature and pollutant mixture experiments at the lowest treatment levels (22°C, 0.05 µg/L, 25%) but at higher treatment levels (24 °C, 0.5 µg/L, 50 % to 28 °C, 50 µg/L, 100 %) there was no difference in snail size between the invasive predator cue and pollutant mixture experiments whereas snails in the temperature experiment were larger (Table S6).

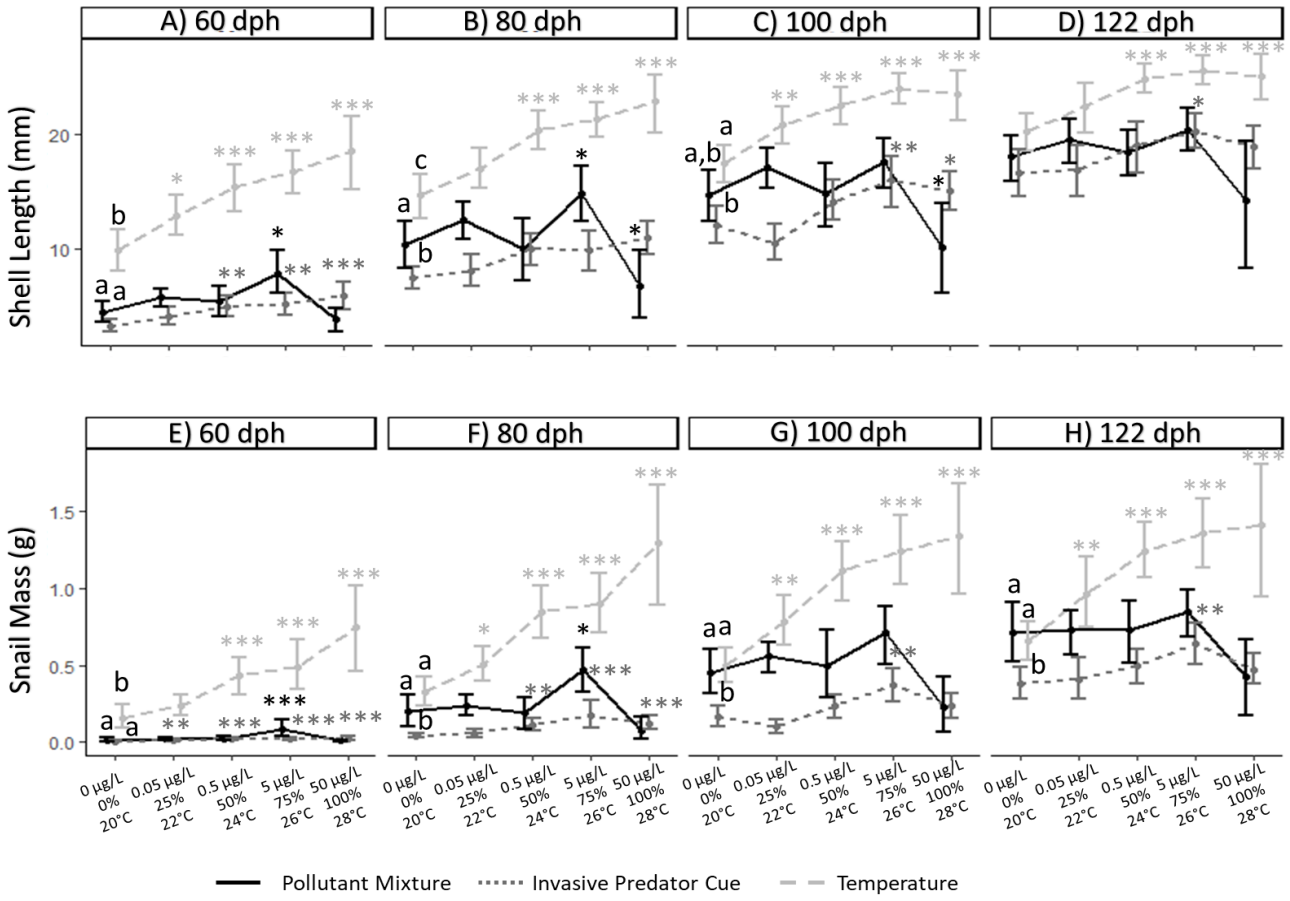


Figure 2: Snail shell length in mm at 60 (A), 80 (B), 100 (C) and 122 (D) days post-hatch (dph) and mass in grams at 60 (E), 80 (F), 100 (G) and 122 (H) days post-hatch (dph) (mean ± 95 % confidence interval) across exposure levels for each stressor. Significance within experiments is denoted by asterisks ('***'p < 0.001; '**' 0.001 < p < 0.01; '*'0.01< p < 0.05). Letters denote differences between experiment controls. For *N* numbers see Table S4.

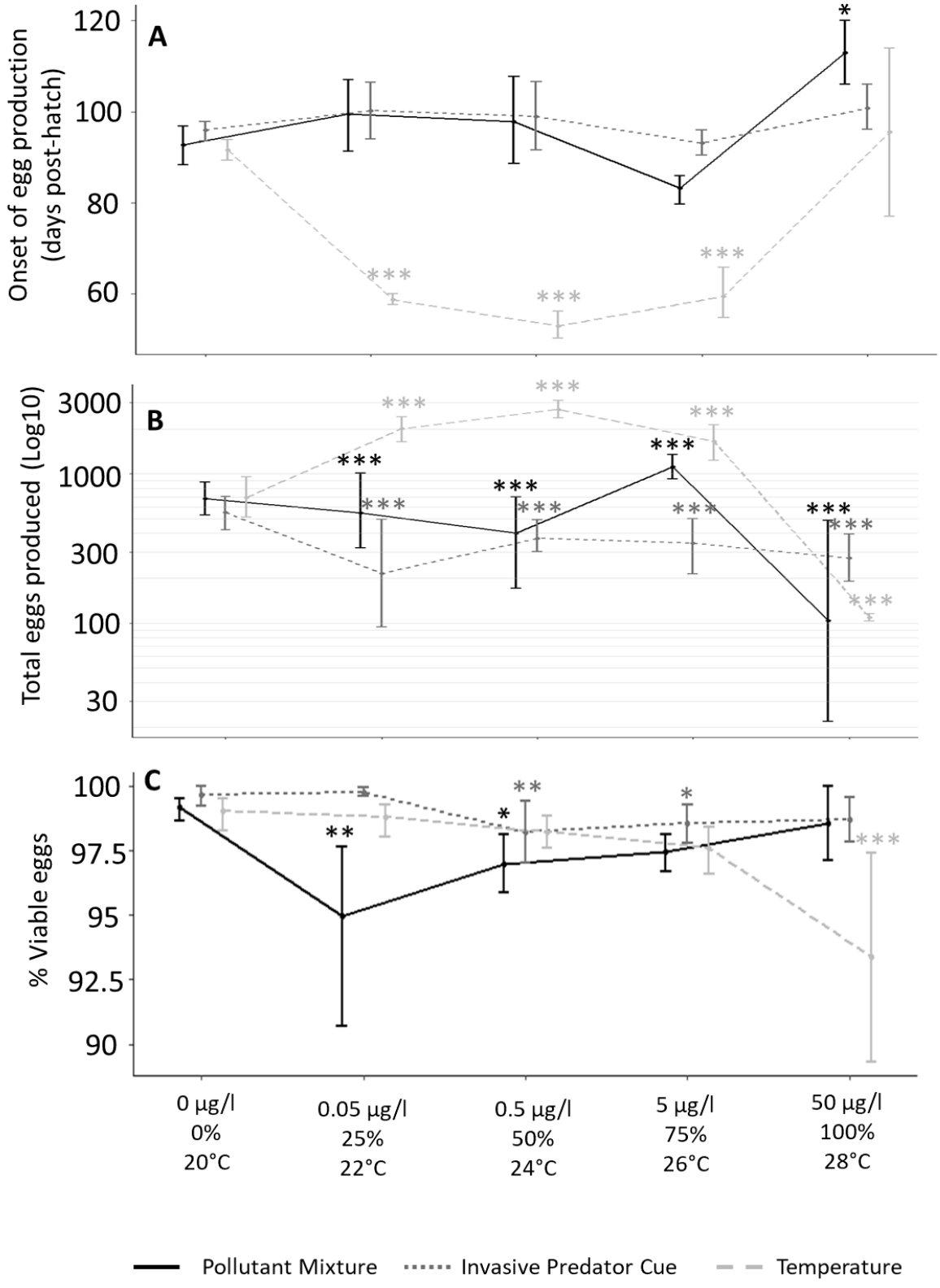
Reproduction

The onset of egg laying occurred earlier in response to higher temperatures (22-26 °C, $p < 0.001$; Fig. 3 A) and occurred later in response to the pollutant mixture exposure (50 µg/L, $p = 0.01$; Fig. 3 A), compared to their respective controls. The total number of eggs produced was higher in response to higher temperatures (22-26 °C; $p < 0.0001$) and lower in response to the invasive predator cue (25-100 %, $p < 0.0001$)and pollutant mixture exposures (0.05, 0.5, 50 µg/L, $p < 0.00017$) except for at 5 µg/L where total eggs were higher ($p < 0.0001$; Fig. 3 B) compared to their respective controls. The proportion of viable embryos was reduced in response to all stressors, with effects occurring at lower exposure levels for the pollutant mixture (0.05 µg/L, 0.5 µg/L; $p < 0.03$) compared to the

310 invasive predator cue (50% - 100%; $p < 0.05$), and the higher temperature (28°C, $p < 0.001$; Fig. 3 C;

311 Table S6). There were no differences in reproductive endpoints observed between experiment

312 controls.



313

Figure 3: A) age at onset of egg production (days post-hatch), B) total eggs produced (Log10 scale) and C) percentage of total embryos that were viable (mean ±95 % confidence interval) across exposure levels for each stressor. Significance is denoted by asterisks ('***'p < 0.001; '**' 0.001 < p < 0.01; '*'0.01< p < 0.05).

Avoidance Behaviour

Avoidance behaviour was observed in response to the highest exposure level for the pollutant mixture (50 µg/L, p = 0.02; Fig. 4) and in response to invasive species predator cue (50 % - 100 %, p < 0.05; Fig. 4). No effects were observed in response to higher temperatures nor between experimental controls.

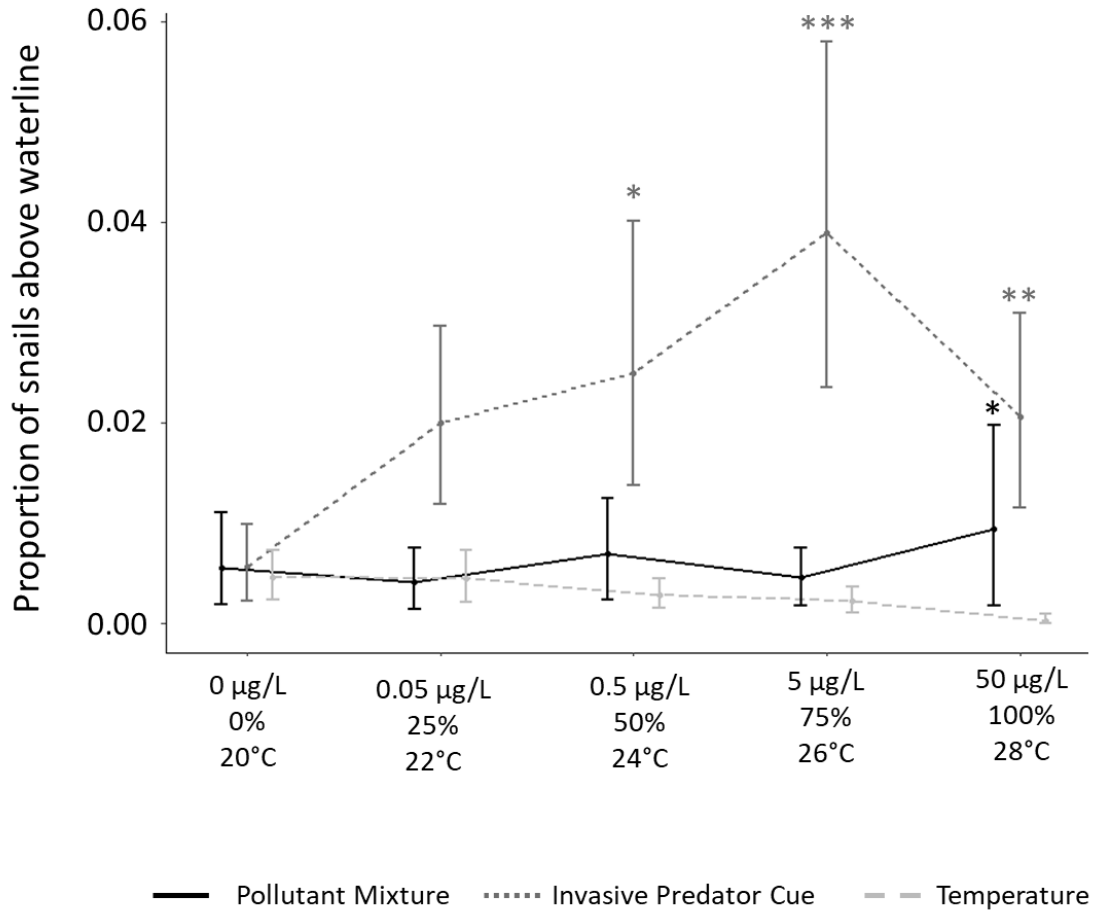


Figure 4: Proportion of snails (mean± 95 % confidence interval) exhibiting escape behaviour (crawled above waterline) after water changes. Significance is denoted by asterisks ('***'p < 0.001; '**' 0.001 < p < 0.01; '*'0.01 < p < 0.05).

Discussion

In this study we set out to compare the impacts of three ubiquitous stressors on life history traits in *L. stagnalis.* We first demonstrate the reliability of our experimental set-up since hatching, post-hatch survival and growth in controls were similar to those previously reported[27,39,40] and did not differ markedly between experiments, except for snail mass in the invasive predator cue control was lower compared with both temperature and pollutant mixture controls (see supplementary Table S6). We also achieved broadly equivalent potencies across our stressor exposure ranges as evidenced by a similar reduction in post-hatch survival in response to the highest exposure level tested for all three stressors. We also sought to investigate whether our ranking of stressors had similarities with the ranking of stressors in FW ecosystems *in situ.* In our experimental set-up, we ranked the pollutant mixture as highest priority for the protection of FW ecosystems since a wide range of adverse effects were observed at much lower exposure levels compared to the other stressors. After pollution, we ranked temperature as the second most important stressor, and the invasive predator cue as the least important in our experimental setup. This was due to invasive predator cue having the least pronounced effects at the lowest exposure levels compared with temperature and the pollutant mixture. Our ranking is supported through pairwise comparisons where the pollutant mixture is shown to induce a higher number of negative effects at lower exposure levels, whereas the temperature stressor had stimulatory effects (growth, reproduction) at lower exposure levels, compared with the other stressors. It should also be noted that the 'equivalent' levels of environmental stressors were hypothesised based on the literature, however, the actual exposure levels of these stressors experienced by wild *L. stagnalis* are not known. Pollution has been cited as one of the most important factors of biodiversity loss in freshwater habitats in several correlational field studies[reviewed in 4,5]. Although these studies focus on nutrient pollution, chemical pollution is also an important factor in freshwater biodiversity loss[reviewed in 21] and often co-occurs with eutrophication[e.g. 41]. Therefore, we provide tentative supporting evidence for the prominent role of pollution in driving negative impacts on FW biota, however, the relative

353 impacts of nutrient *versus* chemical pollutants remain unknown, as do the relative impacts of other
354 unknown interacting factors present at an ecosystem level.

355 In response to higher temperatures, apart from at the highest level tested (28 °C), stimulatory
356 effects were observed. In our experimental set-up, exposure to 24 °C appeared to present ideal
357 conditions for *L. stagnalis*, as stimulatory effects were observed (earlier hatching, faster growth,
358 higher total egg number) in the absence of concurrent toxicity, which is in agreement with a
359 previous shorter term study (111 days from 1 week post-hatch[28]). The toxicity observed in response
360 to the 28 °C condition is in agreement with a previously reported shorter-term exposures of *L.*
361 *stagnalis* (adult 84 day exposure[28]), though this temperature is unlikely to have environmental
362 relevance in the short to medium-term[42]. It was interesting that at the more environmentally
363 relevant temperature of 22 °C, a reduction in survival was also observed, but not at the intermediate
364 temperatures (24 °C and 26 °C). Although we cannot explain this finding, this has been previously
365 reported in *L. stagnalis* exposed to these temperatures for 111 days from 1 week post-hatch[28].
366 Increased water temperature has been also demonstrated to have similar effects in other freshwater
367 species where stimulatory effects of development and growth were observed but with reduced
368 survival (e.g. Odonata[43], Trichoptera[44], Crustacea[45]) upon exposure to predicted global temperature
369 changes.

370 The pollutant mixture negatively affected all endpoints except for time to hatch, in response to at
371 least one of the exposure concentrations and, importantly, negative effects were observed at the
372 lowest exposure concentration (0.05 µg/L). To the authors' knowledge, this is the first time that the
373 effects of exposure to a complex pollutant mixture on full life-cycle endpoints has been reported in a
374 FW invertebrate so comparisons with previous studies are difficult. However, somewhat similar
375 effects have been reported in the embryos of adult *L. stagnalis* exposed over 10 days to a binary
376 pollutant mixture (diquat and nonylphenol), where both hatching rate and success were reduced
377 (222 µg/L diquat + 500 µg/L nonylphenol); although in that study, egg masses were removed daily

from exposure water and reared in clean water so chemical exposure was minimal (< 24 hours)[27]. Similarly, a short-term adult exposure to a multi-component pollutant mixture (17 different pollutants at environmentally relevant concentrations: 0.0003 – 6.36 µg/L) also caused increased growth rates[46], which is similar to the data reported here for the 5 µg/L pollutant exposure but is in contrast to effects observed at 50 µg/L exposure. Although our aim was to investigate the effects of a multi-component mixture at reported environmental concentrations, rather than to assign particular effects to individual components or to investigate interactive effects (e.g. with modelling), it is interesting that some of the components of the mixture have previously been shown to induce similar effects at equivalent exposure levels in single chemical exposures in lymnaeids. For example, copper has been reported to reduce hatching success at 1-32 µg/L[47], which encompasses the nominal concentration used in our highest exposure level (5.38 µg/L in 50 µg/L exposure) where hatching success was also reduced. Nickel has been reported to impact post-hatch survival in juvenile *L. stagnalis* at 0.481 µg/L[48] which is similar to our 5 µg/L pollutant mixture exposure (nickel = 0.52 µg/L in 5 µg/L exposure). However, since the other components of the mixture have not been previously tested on early-life stages of *L. stagnalis* at levels equivalent to those in our mixtures, we cannot determine if the effects we observed were in response to the exposure levels of copper/nickel or other components of the mixture.

A clear stressor-response relationship was observed in response to the invasive predator. While not surprising, since *L. stagnalis* has previously been shown to exhibit avoidance behaviour in response to a native predator[33], this is the first time avoidance behaviour has been reported in response to an invasive predator in this species. These findings are in agreement with reported effects on avoidance behaviour in the FW mollusc *Potamopyrgus jenkinsi* in response to both a native (*Austropotamobius pallipes*) and invasive (*P. leniusculus*) crayfish[30]. Although prey species can fail to recognise a novel predator and elicit any anti-predator response, there is evidence that prey will respond to predator cue from an invasive species if there is a native congeneric of that predator[49]. Therefore, it is possible that snails responded to the invasive predator cue in this study due to recognising a general

404 'crayfish scent' where they may have experienced the native white-clawed crayfish
405 (*Austropotamobius pallipes*) in their natural habitat. In addition, *L. stagnalis* are known to form cue
406 associations[33] and so the response could have been stimulated due to the association of the 'alarm'
407 cue paired with the predator cue. To the authors' knowledge, this specific behaviour has not been
408 previously reported in a gastropod in direct response to a chemical pollutant exposure, however
409 avoidance behaviour towards chemical contaminants has been observed in other aquatic species in
410 the lab such as invertebrates (dipterans, decapods, copepods and annelids) and vertebrates (fish -
411 including three field studies; and amphibians)[reviewed in 50]. Avoidance behaviour was induced in
412 response to the pollutant mixture (50 µg/L) but not in response to the 28 °C temperature exposure,
413 although both these conditions caused significant mortality and reduced total eggs/ embryo viability
414 compared with lower exposure levels indicating greater toxicity. At present, we cannot determine
415 the reason that *L. stagnalis* failed to exhibit avoidance behaviour in response to the high
416 temperature exposure, however, there are several possible explanations for this. Firstly, the high
417 temperature stressor may not have been recognised as a threat since *L. stagnalis* has a large range
418 for thermotolerance[51]. Secondly, the temperature above the water line may not have differed
419 markedly compared to the temperature in the beaker due to the heat of the water bath. Thirdly,
420 increased temperature may have reduced the crawl out behaviour if there was a perceived
421 desiccation risk associated with warmer conditions[52].

422 For chemical risk assessments it is generally assumed that dose-response relationships will follow a
423 sigmoidal curve, though this assumption is being increasingly challenged[e.g. 53]. In our experimental
424 set-up, in response to the pollutant mixture, growth and the proportion of viable embryos did not
425 display a classic sigmoidal dose-response curve but more pronounced effects were observed at
426 lower exposure levels (i.e. a 'non-monotonic' dose-response curve was observed). For example, at
427 80 days post-hatch snails exposed to 5 µg/L were larger compared to controls, whereas those
428 exposed to 50 µg/L were smaller than controls. Interestingly, this type of response has also been
429 documented in juvenile *Physa acuta* exposed to triclosan[54], an endocrine disrupting compound[55],

whereby growth rates increased by 30 % at low concentrations (0.05-1 µg/L) but decreased by 10 % at higher concentrations (> 2 µg/L). These types of responses could be due to general toxicity where a wide range of endpoints are negatively impacted as we have demonstrated in the 50 µg/L exposure (reduced hatching, post-hatch survival, total egg output and proportion of viable embryos). A similar non-monotonic dose-response was observed in embryo viability; however, this is less likely to be due to general toxicity since this effect was only observed in response to the 0.05 µg/L exposure condition. Instead, this type of response may be more specific to alterations in the endocrine system, for which there are many examples of larger effects at low and non-toxic concentrations[53]. Non-monotonic responses have also been reported in other invertebrate species exposed to chemical pollutants such as in the clam *Ruditapes phillipanarum* exposed to cadmium whereby enzyme activity shows a non-monotonic response[56], and in *Daphnia magna* exposed to the biopesticide *Bacillus thuringiensis* whereby survival and immobilisation have a non-monotonic response[57].

In conclusion, using *L. stagnalis* as a model mollusc, our findings indicate that similarly to field derived correlational analyses, pollutants may rank highest for potential to cause harm to FW molluscs with effects observed at our lowest test level which is below the concentrations detected in the environment. We also demonstrate the utility of laboratory-based studies to rank stressors which is an important component in the design effective mitigation strategies to protect FW biodiversity. We also report for the first time the effects of a wide-range of increased temperatures on *L. stagnalis* exposed over the full life-cycle, as well as highly novel findings on the effects of an environmentally relevant multi-component pollutant mixture and invasive predator cue. Although we have demonstrated highly divergent effects dependent on stressor type and exposure level, the presence of multiple stressors in the natural environment are known to interact and can cause synergistic or antagonistic responses[reviewed in58]. Therefore, although ranking stressors by individual exposure effects is important to understand the relative effects, the potential for stressor interactions in the natural environment also requires urgent attention.

Author contributions

Study design: EMM, FO; conducting experiments: EMM; data analysis: EMM, MEA, FO; chemical analysis: MGP, SAT; drafting of manuscript: EMM, KAS, MEA, FO; revision of manuscript: all authors.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Supporting information available

Chemical analysis methodology, shell length measurement schematic, graphical timeline of methods, control /solvent control comparisons, water quality parameters, GLMM test statistics, pairwise comparisons, *N* numbers for growth analysis, sources used to determine exposure levels and median detected environmental concentrations of pollutants used. We would also like to thank two anonymous reviewers whose comments greatly improved the manuscript. This information is available free of charge via the Internet at http://pubs.acs.org.

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