Resilience to heat waves in the aquatic snail *Lymnaea stagnalis*: additive and interactive effects
with micropollutants

Tiina Salo^{1,2,3}*, Christian Stamm⁴, Francis J. Burdon¹, Katja Räsänen^{1,2} & Otto Seppälä^{1,2}

1. Department of Aquatic Ecology, Eawag, Swiss Federal Institute of Aquatic Science and
Technology, Dübendorf, Switzerland

2. Institute of Integrative Biology, ETH Zürich, Zürich, Switzerland

3. Department of Ecology, Environment and Plant Sciences, Stockholm University,
Stockholm, Sweden

4. Department of Environmental Chemistry, Eawag, Swiss Federal Institute of Aquatic
Science and Technology, Dübendorf, Switzerland

* corresponding author: tiina.salo@su.se

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SUMMARY

1. Multiple anthropogenic changes, such as climate change and chemical pollution, threaten the persistence of natural populations. Yet, their potential additive and interactive effects on organismal performance and fitness are poorly understood, thus limiting our ability to predict the effects of the global change.

2. We conducted a laboratory experiment to study the singular and combined effects of experimental heat waves and micropollutants (i.e., low concentration toxicants; henceforth MPs) on the freshwater snail, *Lymnaea stagnalis*. To comprehensively understand physiological and ecological consequences of stress, we studied a broad range of traits from respiration rate to feeding performance and growth. We also determined traits contributing to fitness and immune responses, as these are key traits in determining both organismal fitness and interspecific (e.g. host-parasite) interactions. We tested whether a constant exposure to MPs affect the ability of snails to tolerate heat waves (eight days of 23.5°C), and subsequently to recover from them, and whether the effects are immediate or delayed.

3. We found strong immediate additive effects of both stressors on reproduction, while they synergistically increased respiration and antagonistically decreased food consumption. Moreover, these effects were transient. Although the heat wave increased metabolic rates, individuals did not increase their resource uptake. This caused an apparent imbalance in resource levels – a probable cause for the observed trade-off between immune function and reproductive traits (i.e. phenoloxidase-like activity decreased, whilst reproductive output increased). In addition, exposure to MPs led to a temporarily reduced reproductive output.

4. Our results indicate that even short-term heat waves and low concentrations of chemical pollution can have large, mainly additive impacts on organismal fitness (e.g. altering susceptibility to infections and reproductive output). This suggests that long-term effects of

44 existing stressors and heat waves need to be considered when assessing the resilience of natural
45 populations.

INTRODUCTION

Contemporary simultaneous anthropogenic changes, such as global climate change and pollution, challenge organisms immensely (Millenium Ecosystem Assessment, 2005). One major consequence of climate change is the increased frequency of heat waves (Kirtman *et al.*, 2013), i.e. prolonged periods of unusually hot weather, which can have serious fitness consequences for individual organisms and natural populations. For instance, temperatures that reach the upper thermal limit of organisms (Garrabou *et al.*, 2009), or exposure to increased but (initially) sub-lethal temperatures over an extended period of time (White, Hamilton & Sarnelle, 2015), can reduce survival. Heat waves may also affect internal resource budgets and life history responses of organisms (e.g. Jiguet *et al.*, 2007; Leicht, Jokela & Seppälä, 2013; Ma, Rudolf & Ma, 2015), increase their susceptibility to infections (Thomas & Blandford, 2003; Roth, Kurtz & Reusch, 2010; Seppälä & Jokela, 2011), as well as change behaviour (Dalesman & Rundle, 2010) and species phenologies (Parmesan, 2006).

Responses of organisms to heat waves may, however, depend on the presence of other natural and anthropogenic stressors (Settele *et al.*, 2014), yet we still know very little about the effects of simultaneous exposure to stress, how they interact with increased climatic variability in nature, and the ecological consequences of these interactions. In fact, various anthropogenic stressors such as chemical pollution are becoming increasingly important as drivers of global change (Millenium Ecosystem Assessment, 2005). Multiple stressors may not only behave additively in combination, but can interact, resulting in synergistic or antagonistic effects (Folt *et al.*, 1999). Moreover, as the impacts of stressors may strongly depend on the combination of the stressors in question (e.g. Przeslawski, Byrne & Mellin, 2015), predicting stress responses in nature based on individual impacts of different stressors is problematic. Predicting this is especially challenging

because conducted work focuses on the immediate impacts of extreme temperatures and their interactions with other stressors (Dinh, Janssens & Stocks, 2016). However, some environmental stress effects may only become evident after a lag time due to individual level delayed effects (e.g. Lindström *et al.* 2002) or cross-generational effects (e.g. Mousseau & Fox, 1998; Burton & Metcalfe, 2014). Moreover, stress effects may also manifest themselves as reduced recovery of individuals from stress after conditions become benign again (Dinh, Janssens & Stocks, 2016). Thus, understanding the conjoint and long-term effects of heat waves on organisms is in high demand when predicting the effects of climate change.

Chemical pollution is a pervasive source of anthropogenic environmental stress, especially in aquatic environments (Schwarzenbach *et al.*, 2006). Chemical pollution may affect the ability of organisms to cope with heat waves because, on one hand, toxicity of pollutants may increase with high temperature (reviewed by Holmstrup *et al.*, 2010). On the other hand, resources available for detoxification *versus* growth and maintenance or reproduction under thermal stress may be limited (e.g. Broomhall, 2002). Of particular relevance here is that, despite the improved ecological status of surface waters following improved nutrient retention (i.e. to counteract eutrophication; Jeppesen *et al.*, 2005), chemicals are still continuously released to natural systems through a variety of sources (e.g. municipal wastewater treatment plants, industry and agricultural land use; Schwarzenbach *et al.*, 2006; Wittmer *et al.*, 2010). The concentration of these chemicals can be very low (i.e. in ng l⁻¹ up to µg l⁻¹ range), being then called micropollutants (henceforth MPs). MPs typically consist of a mix of pharmaceuticals, hormones, pesticides, detergents, heavy metals and industrial chemicals (Schwarzenbach *et al.*, 2006). Despite the commonness of these MPs in urban waters, the effects of MPs on organisms, especially in mixes, are still not well quantified (Schwarzenbach *et al.*, 2006; Nunes, 2014). Furthermore, the transformation products

of MPs can be even more toxic than the parental compound and MPs may bioaccumulate in organisms reaching harmful concentrations over time (Boxall *et al.*, 2004). In combination, these compounds can have additive or synergistic effects (Schwarzenbach *et al.*, 2006), can cause changes in the physiology and behaviour of organisms (Nunes, 2014) and weaken the ability of organisms to acclimate and tolerate other abiotic and biotic stressors (Broomhall, 2002).

In this study, we tested experimentally whether organismal responses to heat waves are modified by simultaneous exposure to MPs. We were particularly interested in the potential delayed effects (i.e. delayed effects within individuals or cross-generational effects affecting the hatching success of the next generation) when organisms were exposed to both a heat wave and MPs. We investigated single and combined (additive and interactive) effects of these factors on different organismal traits using *Lymnaea stagnalis* as our model organism. It has been extensively used as a model system to investigate ecological parasitology and immunology (e.g. Dikkeboom *et al.*, 1985; Seppälä *et al.*, 2013), as well as effects of temperature (e.g. Seppälä & Jokela 2011; Leicht, Jokela & Seppälä, 2013) and pollutants (e.g. Coutellec & Lagadic, 2006; Nyman, Schirmer & Ashauer, 2014). Environmental stress, here defined as deviation from optimum conditions resulting in modified organismal performance and/or fitness, can alter energy requirements of organisms and hence resource allocation to different functions (Steinberg, 2012). To quantify the effects of heat wave and MPs on different - possibly competing - components of snail performance, we measured a broad range of traits from respiration rate to feeding performance and growth as well as from fitness components to immune function. We quantified the immediate effects (i.e. directly at the end of the heat wave) of both factors and the delayed effects of heat waves by assessing these parameters both at the end and after the heat wave (i.e. recovery phase). We hypothesized that i) exposure to a heat wave has negative immediate and delayed impacts on

individuals, and that ii) simultaneous exposure to chemicals (i.e. to a mixture of MPs) further reduces the resistance to and recovery from an extreme temperature event through additive or synergistic effects (Folt *et al.*, 1999). We considered antagonistic effects to be unlikely here, where both heat waves and MPs were expected to have negative impacts on organismal responses.

METHODS

Model organism

The great pond snail, *L. stagnalis*, is a hermaphroditic pulmonate gastropod with a wide distribution in stagnant or slowly flowing water bodies in the northern hemisphere. The snails used in this experiment originated from a laboratory population that was started from 88 individuals collected from a shallow pond in Zürich, Switzerland (47°23'N, 8°33'E) during the breeding season 2014. In this region, ponds can have relatively low summer water temperatures (<16°C depending on the hydrology, Salo, pers. obs.). During heat waves, the water temperature can, however, rapidly increase to 20-30°C and can stay high for >14 days (Salo, pers. obs.). Such substantial increases in water temperature have been found to impair *L. stagnalis* performance (Vaughn, 1953; Leicht, Jokela & Seppälä, 2013). While no data on the MP concentrations in pond water in the area exists, MPs are common in fresh water systems in the central Europe (e.g. Wittmer *et al.*, 2010; Munz *et al.* 2016).

Because these snails may engage in multiple matings (Nakadera *et al.*, 2017) and can store allosperm for over two months after the last copulation to be used for cross-fertilization (Nakadera, Blom & Koene, 2014), genetic variation in the laboratory population should adequately reflect genetic variation in the population of origin. To minimize unwanted

transgenerational effects arising from variation in environmental conditions in the wild, the population was kept in laboratory for three generations prior to the experiment. The laboratory population was kept in a large (800 l) aerated tank with slowly flowing water at ca. 18 °C. Snails were fed three times a week with fresh lettuce and pulverized *Spirulina*.

Experimental design

The experimental design was a fully randomized factorial design consisting of a combination of two temperature (heat wave and no heat wave treatment) and three chemical treatments (MPs, technical control and control, Fig S1). Each of the six treatment combinations was sampled in three separate phases: at the end of an eight-day heat wave, eight days after the end of the heat wave (recovery phase 1) and 16 days after the end of the heat wave (recovery phase 2), with 18 replicate snails for each treatment combination and phase (n=54 per treatment combination). Heat wave was designed to last for eight days, which is close to the average length (8.4 days) of natural heat waves in Western Europe (Meehl & Tebaldi, 2004). Each snail was only sampled once (i.e. snails sampled at the end of the heat wave phase were removed from the experiment at this stage, whereas snails that were kept until the second recovery phase were not sampled during earlier phases).

Initially, 324 adult snails were haphazardly chosen from the laboratory population for the experiment. The experiment was conducted in two separate walk-in climate chambers at the laboratory facilities of Eawag, Dübendorf, Switzerland. Initially, all snails were kept in one climate chamber (at $14.0 \pm 1.5^{\circ}\text{C}$) and only divided into two chambers to mimic a heat wave ($23.5 \pm 2^{\circ}\text{C}$) for half of them (see below). The snails were kept individually in 200 ml glass containers in 14/10 h light/dark cycle at $14.0 \pm 1.5^{\circ}\text{C}$ and fed *ad libitum* with fresh organic

lettuce. One third of the snails (n=108) were kept in aged non-chlorinated tap water spiked with MPs (MP treatment, see below) and one third (n=108) in aged tap water mixed with low concentration of MeOH (technical control). The technical control (TC) was needed to control for the effects of MeOH that was used to solubilize chemicals in the MP treatment. The MeOH concentration in the TC was same as in the MP treatment (i.e. 4.20 mg l⁻¹). In addition, one third of the snails (n=108) were assigned to a second control (C) consisting of aged tap water without any added chemicals (Fig. S1). However, as the responses in the two control treatments did not differ significantly (except for ca 2 % higher food consumption rate in C compared to TC treatment during the second recovery phase and ca 74 % lower specific growth rate in TC compared to C in the 14°C treatment during the first recovery phase), results are presented only for the MP and TC treatments (n=216, see Table S1 for the comparisons between the TC and C). Individuals experienced the same chemical treatment throughout the experiment. The initial shell length (size) ranged from 21.3 to 38.7 mm (mean 28.4 mm) and did not differ between any of the chemical and temperature treatment combinations (separate two-way univariate permutational analysis, $p > 0.05$ for both chemical and temperature treatments and for the interaction).

As we were interested in the general impacts of MPs (rather than effects of any specific MP), and as MPs typically occur as a complex mixture in natural aquatic environments (Schwarzenbach *et al.* 2006), the MP treatment consisted of spiking with a standardized mixture of chemicals (Table 1) similar to the mix described in Stamm *et al.* (2016). These chemicals were chosen because they are commonly observed in aquatic systems impacted by wastewater effluent and, as biologically active compounds, have the potential to cause undesired effects in aquatic organisms (Schwarzenbach *et al.*, 2006). To ensure constant exposure to MPs and sufficient water quality, water was changed every second day in all experimental units. To analyse whether MP

concentrations were affected by the temperature treatments or by the presence of snails, water samples from three haphazardly selected experimental units and control units without snails were collected on three occasions during the heat wave (see below) both after and before the water change. The concentrations of majority of the chemicals did not change over time, but two chemicals degraded notably ($> 40\%$ lower concentrations; diclofenac and diazinon) and three chemicals degraded somewhat ($< 30\%$ lower concentrations; benzotriazole, metformin and citalopram) in two days (Table 1; see supplementary material for details on the MP analyses).

The snails were maintained in their initial chemical treatments for 16 days at 14°C , at which point half of the snails from each chemical treatment were exposed to an experimental heat wave ($23.5 \pm 2^{\circ}\text{C}$) for eight days. In the heat wave treatment, temperature of the climate chamber was increased from 14°C to the target temperature of 23.5°C , increasing the water temperature by ca 1°C h^{-1} . Similar temperature increases have been quantified in small natural ponds during warm summer days (Salo, pers. obs.). Eighteen haphazardly selected individuals (or less in case of mortality) from each temperature and chemical treatment combination were sampled at the end of the heat wave to assess their single and combined effects on the different response variables (see below). For the remaining snails (i.e. 36 per treatment combination), the water temperature was slowly decreased back to 14°C during ca 10 h and the snails were allowed to recover from the heat wave for either eight (recovery phase 1) or 16 (recovery phase 2) days. These snails were then sampled in a similar manner at the end of their respective recovery phase.

Response variables

Survival of all snails was checked every second day, but as only 14 individuals (4.3 %) died (with no apparent pattern across the treatments), survival was not further analysed and data collected from these individuals were excluded from any analyses.

Metabolism

Respiration rate of each individual was assessed on the second last day of the respective experimental phase (Fig. S1b). Snails were placed in separate 185 ml plastic containers filled with water from their respective chemical treatment and incubated at temperatures corresponding to their experimental treatment at the time of the measurement. Magnetic stirrers were deployed in each container to ensure constant mixing of the water. Snails were allowed to acclimate in the incubation container for 10 minutes prior to any measurements. Respiration was measured using a Fibox 4 system (PreSens Precision Sensing GmbH, Regensburg, Germany) with optical oxygen sensors. Oxygen concentration (g O_2) was then recorded at least 25 times during a 15 minutes period. Respiration rates ($\text{mg O}_2 \text{ h}^{-1}$) were then calculated from periods with linearly changing ($R^2 > 0.85$) oxygen concentration. Measurements with lower linear fit (in total 11 of the 310 measured individuals) were excluded from the results.

Food consumption

Food consumption was assessed for each individual snail during the last day of the respective experimental phase (Fig. S1b). The snails were offered an approximately equal amount (ca 1.3 g) of fresh organic lettuce, and consumption (g blotted fresh weight of lettuce, FW) was calculated as the change in food weight during 24-27 h. As lettuce was decomposing faster in the high temperature treatment, the initial and final weight of three replicates of lettuce submerged in

water without a snail were measured to compensate for extrinsic impacts on lettuce weight, and food consumption was calculated according to Equations 1 and 2,

$$FW_{corrected} = \frac{FW_{initial} \times FW_{CF}}{FW_{CI}} \quad \text{Equation 1}$$

$$Food\ consumption = \frac{FW_{corrected} - FW_{final}}{h} \quad \text{Equation 2}$$

where $FW_{corrected}$ is the corrected start weight, $FW_{initial}$ and FW_{final} are the initial and final FW of the lettuce offered to the snail, respectively, FW_{CI} and FW_{CF} are the initial and final weight of the control lettuce, respectively, and h is the feeding time in hours.

Growth

To assess growth, snail shell length was measured to the nearest 0.1 mm both before and at the end of the experimental phase at which it was sampled. As snail growth typically follows a power function (Seppälä *et al.*, 2013), the specific growth rate (SGR) of each snail was assessed according to Equation 3,

$$SGR = \frac{(\ln S_2 - \ln S_1)}{\Delta t} \quad \text{Equation 3}$$

where S_2 and S_1 are shell length at the end of and before the experimental phase, respectively, and t is the time between the measurements in days.

Reproduction

The reproductive output was measured for each snail as i) whether or not it produced eggs, and ii) if it did, the total number of eggs oviposited (i.e. fecundity). *Lymnaea stagnalis* produces multiple clutches sequentially and hence all egg clutches oviposited by a given snail during a given experimental phase were sampled. Each egg clutch was placed on a luminant surface and photographed with a digital camera to count number of eggs (i.e. reproductive output). The

number of eggs in each digital image was counted manually with the help of Cell Counter-plugin in Image J software (ImageJ 1.49k, Wayne Rasband, National Institute of Health, USA). Common garden approach was used for later assessment of hatching success of embryos (i.e. number of hatched offspring/total number of eggs per individual), and the clutches were placed in separate 200 ml plastic containers containing aged tap water and maintained at $14 \pm 1.5^{\circ}\text{C}$. Finally, to assess the importance of maternal chemical and temperature exposure on hatching success of embryos and total number of offspring, number of hatchlings was recorded from the collected egg clutches weekly for 12 weeks. The water for embryos was changed weekly.

Immune defence

Immune defence parameters were measured from snail haemolymph using standard procedures and consisted of phenoloxidase (PO)-like activity and antibacterial activity. PO is used as an oxidative defence against parasites (Söderhäll & Cerenius, 1998), while antibacterial activity is a defence against invading microorganisms (Imler & Bulet, 2005). Haemolymph samples were collected at the end of the respective experimental phase for each snail (Fig. S1b), and analysed implementing methods in Leicht, Jokela & Seppälä (2013) (see Supplementary material for detailed description of the method).

Statistical analyses

All statistical analyses were conducted using permutational multivariate and univariate analysis in PERMANOVA+ (v. 1.0.3) package in PRIMER 6 (v. 6.1.13, Auckland, New Zealand). Due to the strong effects of temperature on organismal responses during the heat wave-phase, the effects of experimental phase and temperature would not have been possible to tease apart in an analysis incorporating time as a fixed factor. Thus, results from the three different time points (heat wave,

eight days of recovery and 16 days of recovery) were analysed separately. This approach allowed comparing responses between treatments and treatment combinations within experimental phases according to the aims of the study.

As body size can affect many of the measured traits (e.g. respiration, growth rate, reproduction), we used geometric mean of shell lengths for the respective measurements as a covariate in all the analyses, except for the analysis of initial shell length. A full PERMANOVA model with covariate (size), temperature treatments (exposure to constant 14°C versus temporary heat wave 23.5°C), chemical treatments (MP, TC), interaction between temperature and chemical treatments and interactions between the covariate and the main factors was always analysed first to inspect the homogeneity of the within group regression slopes. If no significant interactions between the covariate and main factors were found, or the heterogeneity of slopes was negligible when inspected with scatterplots (Quinn & Keough, 2002), the reduced model (without covariate-main factor interactions) was run. Analyses with significant results at $\alpha = 0.05$ were followed by permutational pairwise testing. Resemblance matrixes were based on Euclidean distance and PERMDISP analyses and MDS plots were used to inspect the dispersion of the data. In case of heteroscedastic dispersion, data were square root-transformed to improve the dispersion. The data were normalized prior to the analysis. All analyses were run with type I SS and with 9999 permutations.

To study the general treatment effects on the composite phenotype, within phase analyses were always initiated with multivariate permutational analysis. Only variables that were measured at the individual snail level (i.e. respiration rate, food consumption, specific growth rate, number of produced eggs, PO-like activity and antibacterial activity) were included in the multivariate

assessment. Thus, variables that were also dependent on the performance of the second generation (i.e. embryonic hatching success and total number of hatched offspring) or that were binomial (i.e. whether or not an individual reproduced), were only assessed in univariate analyses. To study the treatment effects on specific traits in more detail, multivariate analyses were followed by separate two-way permutational univariate analyses for each separate response variable. Apart from the dependent variables in the multivariate analyses, all data were treated as independent, as different haphazardly selected snails were measured at each sampling occasion. Results for whether the individuals did or did not reproduce were analysed using binomial data, and only snails that oviposited eggs were included in the analysis of fecundity.

As stress effects can range from additive to antagonistic and synergistic, we calculated interaction effect sizes according to Jackson *et al.* (2016). These were calculated for all the studied response variables (with an exception of the binomial variables) and separately for each experimental phase. This method estimates the difference between the observed net impact and the expected (additive) effect of combined stressors. Shortly, we estimated the expected additive effect (X_e) according to equation 4,

$$X_e = (X_{HW} - X_C) + (X_{MP} - X_C) + X_C \quad \text{Equation 4}$$

where X_{HW} , X_{MP} and X_C indicate the mean of responses in the heat wave, micropollutant and control (i.e. constant temperature, no MPs) treatments, respectively. The expected additive effect and the observed net impact were then used to calculate Cohen's d according to Nakagawa and Cuthill (2007) and Jackson *et al.* (2016). An effect was evaluated significant (antagonistic or synergistic) when the estimated 95% confidence intervals did not cross zero, while the non-significant effects were considered additive.

RESULTS

Multivariate responses

Temperature contributed over four times more to the differences in multivariate trait expression compared to chemical exposure during the heat wave (Table 2). The observed temperature effects were transient and the contribution of the heat wave treatment, in terms of proportion of variance explained, decreased with time (from 12.3% during the heat wave to 0.4% after 16 days of recovery). Contrastingly, the impacts of MPs tended to increase with time (from 2.6% after the heat wave to 3.7% after 40 days of exposure at the end of the experiment) and constant-long term exposure to MPs had an impact on the composite phenotype (Table 2). Interactive impacts of temperature and chemical exposure on the composite phenotype were first observed during the recovery phase and explained 4.1% of the variance (Table 2). However, these effects on the multivariate trait expression were weak and pair-wise comparisons did not reveal any significant differences between the different temperature-pollutant combinations.

Metabolism

Respiration rate of snails increased over two-fold during the experimental heat wave compared to the constant 14°C (Table 2, Fig. 1a), suggesting that metabolic rate of snails also increased. Temperature and chemical treatment had interactive synergistic effects on respiration rate (Table 2, Fig. 2) as exposure to MPs modified the respiration rate during exposure to the heat wave (Fig. 1a). More specifically, of the individuals exposed to heat wave, the ones that were simultaneously exposed to MPs tended to have ca 20% higher respiration rate than the individuals that experienced no MPs, although not significantly so (pair-wise permutational post hoc comparisons: $p=0.051$). Meanwhile, the respiration rates in the constant temperature treatments were similarly independent of the chemical treatment (pair-wise permutational post hoc

comparisons: $p>0.3$). Metabolic rates rapidly re-adjusted after the heat wave and no differences between temperature treatments were observed in the respiration rates in either of the recovery phases (Table 2, Fig. 1a).

Food consumption

During the heat wave phase, temperature and chemical treatments had no significant main effects but did have an interactive antagonistic effect on food consumption (Table 2, Fig. 1b, Fig. 2).

This effect arose because the combination of a heat wave and MPs tended to reduce food consumption by 30 % compared to a heat wave without MPs. However, no differences between the individuals exposed to MPs and the individuals that did not experience MPs were found within either of the two temperature treatments in the post hoc comparisons. During the first recovery phase, neither previous exposure to a heat wave nor chemical treatments affected food consumption, while during the second recovery phase a significant antagonistic interaction of temperature and chemical exposure was observed (Table 2, Fig. 1b, Fig. 2). This was caused by 55-fold higher feeding rates in the individuals that had experienced the heat wave, but were not exposed to MPs compared to the individuals exposed to both the heat wave and MPs (who ceased to eat; pair-wise permutational post hoc comparison: $p=0.0019$). Additionally, food consumption rates in the individuals that were not exposed to MPs that had experienced the heat wave were nine-fold higher compared to the constant temperature, no MPs treatment combination (pair-wise permutational post hoc comparison: $p=0.025$).

Growth

During the heat wave phase, neither temperature nor chemical treatments had significant effects on specific growth rate (Table 2, Fig. 1c). Previously experienced temperature conditions and

chemical treatment did, however, have an interactive antagonistic effect on specific growth rate during the first recovery phase (Table 2, Fig. 1c, Fig. 2). This interaction arose because of individuals in the constant temperature treatment that did not experience MPs had a slower specific growth rate than individuals exposed to MPs (pair-wise permutational post hoc comparisons: $p=0.042$), whereas individuals that had been exposed to a heat wave showed no significant effect of the chemical treatment ($p=0.086$). This effect was likely stochastic as the specific growth rate in the TC treatment here was much lower also compared to the C treatment (see materials and methods –section). These interactive treatment effects were short-lived, however, as there were no significant treatment effects on specific growth rates during the second recovery phase (Table 2, Fig. 1c).

Reproduction

Most individuals (92.3%) that survived to the end of the experiment reproduced, without any apparent treatment effects on whether or not an individual reproduced (Table 3). During the second recovery phase of the experiment, however, individuals that had been exposed to the heat wave were more likely to reproduce than the ones that were constantly kept at 14°C (97.2% vs. 79.4%, respectively, Table 3).

During the heat wave phase, fecundity (total number of eggs produced) of individuals that did reproduce was affected additively both by temperature and chemical treatment (Table 2, Fig. 2, Fig. 3a). Overall, fecundity was ca 1.5 times higher during the heat wave compared to constant 14°C, but decreased quickly thereafter. There were no significant effects of the previously experienced heat wave during either of the recovery phases (Table 2, Fig. 3a). MPs had negative impacts on reproduction during the heat wave phase, with about 25% lower egg production in the

MP than in the no-MP treatment (Fig. 3a). Exposure of mothers to the heat wave and/or MPs did not affect the hatching success (%) of their embryos significantly (Table 3). Thus, with the higher number of eggs produced during the heat wave, the number of hatched offspring was increased by 50% by maternal exposure to the heat wave compared to constant temperature, but only transiently (Table 3, Fig. 2b).

Immune defence

During the heat wave phase, PO-like activity was 36% lower in the heat wave than the constant temperature treatment (Table 2, Fig. 3c). There were no significant treatment effects on PO-like activity during neither of the recovery periods (Table 2). Antibacterial activity was kept at a relatively constant level and neither temperature nor chemical treatment affected it significantly (Table 2, Fig 3d).

DISCUSSION

Our study shows that heat waves can have strong effects on different components of organismal fitness, which is in accordance to previous studies (e.g. Jiguet *et al.*, 2007; Leicht, Jokela & Seppälä, 2013; Ma, Rudolf & Ma, 2015). However, our study also demonstrates that MPs have the potential to exert negative effects on organisms - despite their low concentrations in natural systems (Schwarzenbach *et al.*, 2006). We found immediate individual and additive impacts of the heat wave and MPs, with evidence for some interactive (antagonistic and synergistic) effects (summarized in Fig. 4). Specifically, exposure to a heat wave increased metabolic rates and reproductive output, but decreased oxidative immune defence. In contrast, exposure to MPs decreased reproductive output. However, these effects were mainly transient and suggest rapid recovery from heat waves and quick acclimation to chemical pollution.

Metabolic balance

Metabolic rates of ectothermic organisms generally increase exponentially with temperature over normal operating ranges (Brown *et al.*, 2004). To keep up with the increased metabolic demands (i.e. energy requirements), organisms should increase feeding rates when the temperature of their environment rises (Brown *et al.*, 2004). However, if resource supply is inadequate or foraging is inefficient, high temperatures may lead to decreased growth rates, reduced reproduction and starvation (Lemoine & Burkepile, 2012; see Fussman *et al.*, 2014 for a meta-analysis across variety of species and ecosystems). We observed increased respiration rates of individuals during the experimental heat wave, with synergistic impacts when simultaneously exposed to MPs. This indicates that metabolic demands increased. Snails, however, did not increase food consumption during the heat wave treatment. Moreover, snails exposed to both high temperature and MPs antagonistically reduced their feeding rate. Metabolic imbalance can lead to trade-offs between important fitness traits (VanNoordwijk & DeJong, 1986; Reznick, Nunney & Tessier, 2000) or even reduce the rate of all energy-demanding processes due to metabolic depression (Dinh, Janssens & Stocks, 2016). In fact, the observed discrepancy between resource demand and uptake during the heat wave (and when concurrently exposed to MPs) reduced investment in energy-demanding processes (i.e. immune function, growth and reproduction; see below). Further, previously experienced heat wave and continuous exposure to MPs antagonistically decreased both respiration rate and food consumption during the second recovery phase. These delayed impacts could indicate metabolic down-scaling due to acclimation to a lower temperature regime in the presence of MPs. The timing of these responses suggests a short delay in the acclimation process.

Reproduction

Environmental factors that influence the nutritional state of individuals can affect maternal investment (number and quality of eggs) (e.g. van Noordwijk and DeJong, 1986; Jiguet *et al.*, 2007; Ma, Rudolf & Ma, 2015) and, subsequently, offspring performance (e.g. Jiguet *et al.*, 2007; Ma, Rudolf & Ma, 2015). In our study, temperature and pollutants had additive effects on fecundity (i.e. number of eggs produced): exposure to MPs resulted in a decrease in fecundity, while the heat wave increased fecundity. We found a lower relative reproductive investment of snails during the first phase (i.e. temperature manipulation) of the experiment when exposed to chemical stress. This suggests that exposure to MPs can have an initial negative impact on reproductive fitness, but that physiological acclimation (i.e. seen here as an increase in relative reproductive investment after the first phase) may allow individuals to minimize any further negative impacts on their fitness. Heat waves tend to shorten the life span of an organism considerably (e.g. Ma, Rudolf & Ma, 2015; Pitt & Kaeberlein 2015), with a simultaneous cost to life time fitness. As survival and reproduction are typically traded off (Williams, 1966), at least under resource-limited conditions (e.g. van Noordwijk & DeJong, 1986; Reznick, Nunney & Tessier, 2000), the observed increase in fecundity during the heat wave could reflect terminal investment (Williams, 1966). The increase in reproduction during the heat wave, concomitant to the observed imbalance between metabolic demand and food consumption, likely depleted energy resources available for investment in other traits (see below). In our laboratory experiment, snails were fed *ad libitum* which may have weakened any energetic constraints. However, during periods of limited food availability or quality (as typical in nature), the discrepancy between metabolic demand and food consumption should become even more pronounced, thus affecting the quality and quantity of eggs and the severity of trade-offs between traits (VanNoordwijk & DeJong, 1986; Reznick, Nunney & Tessier, 2000). Accordingly, Adamo

et al. (2012) observed increased reproductive rates in crickets during a heat wave when resource availability was high, while limited resources led to reduced reproductive output. While limited resources often result in lower individual fecundity (McGinley *et al.*, 1987; Adamo *et al.*, 2012), they may simultaneously increase the parental investment in offspring quality (McGinley *et al.*, 1987). While we did not quantify egg size in this experiment, the percentage of embryos that hatched was similar across the different environmental conditions. This strongly suggests that while the heat wave and MPs affected the maternal investment in egg quantity under *ad libitum* conditions, they had less effect on the amount or quality of resources invested per egg.

Immune defence

Immune defence can be affected by many extrinsic factors, such as temperature (Thomas & Blandford, 2003; Martin *et al.*, 2010), resource level (Stahlschmidt *et al.*, 2015) and pollutants (Martin *et al.*, 2010). Heat waves may critically impair invertebrate immunocompetence (e.g. in butterflies: Karl *et al.*, 2011, damselflies: Dinh, Janssens & Stocks, 2013, isopods: Roth, Kurtz & Reusch, 2010, snails: Seppälä & Jokela, 2011; Leicht, Jokela & Seppälä, 2013). Similarly to these studies, we found impaired immune defence in the heat wave treatment in terms of lower PO-like activity. This concurrent de-activation of oxidative immune defences and the observed increase in fecundity (i.e. number of produced eggs) suggests a trade-off between immune and reproductive traits after a longer warming event due to limited internal resources. Investment in reproduction over immune defence can increase organismal fitness if infection risk is low. However, since parasites reduce host survival (Minchella, 1985) and/or fecundity (Minchella, 1985; Lafferty & Kuris, 2009) the observed responses to high temperature may reduce the residual reproductive value of individuals if heat waves take place, for instance, during epidemics. Thus, prevalence of parasites in the host population may define the selection on snail

responses to heat waves, strong responses being favoured by selection with low infection risk and weak responses favoured when parasites are common.

General implications and conclusions

Climate change related ecological research has increased greatly during the last two decades (Woodward and Perkins, 2015). However, although much attention has been given to the individual effects of increased temperature on biological responses (e.g. Angilletta, 2009), we still know little about how temperature in combination with other global change related stressors affect organisms (Settele *et al.*, 2014). Our study shows that that heat waves and MPs can have strong individual and combined effects on phenotype and fitness of organisms (summarized in Fig. 4). In line with our expectations, we observed strong additive effects of heat wave and MPs on fecundity, while the interactive synergistic and antagonistic effects of the studied stressors were less pronounced on any of the studied traits, and only affected food consumption, respiration and growth transiently. The lack of further interactive impacts could be either due to acclimation to the altered environment or alternatively due to a high tolerance to stress in our study organism. It has been suggested that freshwater organisms are generally adapted to rapid changes in the environment and, thus, potentially selected for stress tolerance and plasticity (Jackson *et al.*, 2016). If the observed plasticity is adaptive (i.e. mean phenotypic response changes towards the new optimum) it will enhance organismal function and survival in the changing world (Ghalambor *et al.*, 2007).

Both the individual and interactive effects were more firmly detected when several traits were assessed simultaneously in multivariate space. Studying a combination of traits also enabled determining the underlying metabolic imbalance as a probable cause for the observed responses,

increasing mechanistic understanding of stress effects on organisms. However, as our study was conducted in standardized and benign laboratory conditions, we may underestimate the negative impact on the fitness of individuals and populations in nature. In the complex natural environment, organisms are continuously exposed to parasites, predators and resource limitation, whereby any effects of single or combined abiotic stressors are likely magnified in natural ecosystems. Any impacts of temperature and pollutant stress on individual phenotype may thus affect population size and demography either directly or indirectly. Future studies should therefore study the composite phenotype stress responses (from physiology to fitness) in a combination of ecologically relevant resource conditions.

MPs were expected to have modest effects on our study organism as the concentrations of the pollutants were kept low. Previous studies looking into the effects of simultaneous exposure to high temperature and pollutants have reported higher frequency and impact of synergistic effects of these stressors (summarized by e.g. Holmstrup *et al.* 2010). The concentrations of pollutants in these studies were, however, higher than what is normally observed in natural environments (Munz *et al.* 2016). The magnitude of synergism may thus depend both on the chemical concentration and the exposure temperature (e.g. for diazinon, see Osterauer & Köhler, 2008). In this study, contrastingly to our expectations, antagonistic effects of chemical and temperature stress were more frequently observed compared to synergistic effects. Whether this simply reflects the dose-response ratio, depends on the specific chemical interactions in the mixture (Pomati *et al.*, 2008) or is species-specific, warrants further studying. Concentrations used in previous experiments are commonly too high and ecologically irrelevant to conduct relevant comparisons with our observations. Thus, more research on the mechanistic effects of mixtures of MPs on organisms is needed.

546

547 While the differences in organismal responses to mixtures of MPs among species still remain
548 largely to be discovered, altered species composition in natural populations close to waste water
549 treatment plants suggest radical differences in tolerance to MP mixtures among species (e.g.
550 Englert et al. 2013; Stalter et al. 2013; Münze et al. 2017). Gastropods, for example, may be
551 relatively tolerant to pesticides, while similar concentrations experienced by other, more sensitive
552 species may have detrimental effects on survival and population size (Hua & Relyea, 2014).
553 Similarly, due to differences in temperature tolerance, resilience to heat waves differs between
554 organisms and populations (Parmesan, 2006). While our study organism showed in general high
555 plasticity and resilience in response to multiple stressors, lower plasticity in other organisms or
556 populations could lead to strongly reduced fitness. Thus, the relatively high resilience of *L.*
557 *stagnalis* in this study should not be taken as an indication of modest effects of MPs, heat waves
558 and their interactions on aquatic organisms, but rather an expression for the need for comparable
559 laboratory experiments to increase mechanistic understanding of species' sensitivity to
560 anthropogenic stress, especially when exposed to several stressors simultaneously.

561

562 In conclusion, our study on *L. stagnalis* clearly shows that even short-term heat waves (here eight
563 days) and low concentrations of chemical pollutants can have negative effects on the composite
564 phenotype. Specifically, we found that heat wave-induced metabolic imbalances can lead to
565 trade-offs between competing functions such as reproduction and immune defence, whereas
566 constant pollution load primarily reduced fecundity. Our evidence for a quick recovery of
567 individuals from the heat wave and rapid acclimation to long-lasting stress (here MPs) are in line
568 with recent work that illustrate that much of organismal adaptive responses to climate change
569 reflect phenotypic plasticity (Merilä & Hendry, 2014). Our findings of stress effects on traits

570 closely linked to fitness emphasize the ecological impacts that anthropogenic stressors can have
571 on organismal functioning and individual life histories in the face of the ongoing global change.

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Table 1. Chemicals used in the micropollutant treatment and some physicochemical properties of these. Start concentration indicates the average start concentrations before the two-day incubations, while the other concentrations indicate the final concentration when incubated with or without snail in 14°C or 23.5°C, respectively. *concentration below detection limit, values from calculations. ^S, ^T and ^{SxT} indicate significant difference in final concentration between snail and control treatments, between temperature treatments and interaction between these, respectively. ^aestimated (EPISuiteTM; <http://www.chemspider.com>; accessed 3 May 2016) ^bexperimental + estimated (EPISuiteTM; <http://www.chemspider.com>; accessed 3 May 2016) ^c(Tomlin, 2003)

Group	Chemical	Start conc. (µg/l)	Conc. with snails (µg/l) in 14 °C	Conc. with snails (µg/l) in 23.5°C	Conc. control (µg/l) in 14 °C	Conc. control (µg/l) in 23.5 °C	CAS no	Log Kow	Water solubility (mg/L)
Pharmaceuticals	Amisulprid	0.100 ± 0.003	0.095 ± 0.001	0.101 ± 0.002	0.096 ± 0.002	0.097 ± 0.002	71675-85-9	1.11 ^a	542.5 (25°C) ^a
	Atenolol	0.224 ± 0.003	0.210 ± 0.004	0.204 ± 0.004	0.213 ± 0.004	0.207 ± 0.008	29122-68-7	0.16 ^b	685.2 (25°C) ^b
	Carbamazepine	0.288 ± 0.019	0.267 ± 0.007	0.258 ± 0.008	0.277 ± 0.006	0.282 ± 0.012	298-46-4	2.45 ^b	17.66 (25°C) ^b
	Citalopram	0.024 ± 0.001	0.021 ± 0.001	0.021 ± 0.002	0.022 ± 0.001	0.018 ± 0.002	59729-33-8	3.74 ^a	31.09 (25°C) ^a
	Clarithromycin	0.035 ± 0.001	0.034 ± 0.001 ^{S,T}	0.029 ± 0.002	0.035 ± 0.001	0.033 ± 0.001	81103-11-9	NA	NA
	Diclofenac	0.230 ± 0.003	0.147 ± 0.018 ^S	0.112 ± 0.010	0.231 ± 0.002	0.209 ± 0.020	15307-86-5	4.51 ^b	4.52 ^b
	Fexofenadine	0.083 ± 0.002	0.084 ± 0.001	0.083 ± 0.001	0.084 ± 0.001	0.085 ± 0.001	83799-24-0	NA	NA
	Iopromide	1.510 ± 0.040	1.577 ± 0.025	1.667 ± 0.047	1.590 ± 0.017	1.597 ± 0.040	73334-07-3	NA	NA
	Metformin	6.360 ± 0.320	4.982 ± 0.796	5.452 ± 0.697	4.813 ± 0.669	4.638 ± 0.884	657-24-9	-1.40 ^a	1'000'000 (25°C) ^a
	Valsartan	0.335 ± 0.011	0.282 ± 0.005 ^{SxT}	0.335 ± 0.010	0.287 ± 0.005	0.283 ± 0.013	137862-53-4	3.65 ^a	1.41 (25 °C) ^a
Estrogenic hormone	Estradiol*	0.0003					50-28-2	4.01 ^b	81.97 (25°C) ^b
Herbicide	Diuron	0.058 ± 0.002	0.051 ± 0.002	0.054 ± 0.003	0.055 ± 0.003	0.043 ± 0.004	330-54-1	2.85 ^c	42 (25 °C) ^c
Fungicide	Tebuconazol	0.009 ± 0.001	0.008 ± 0.001	0.011 ± 0.002	0.009 ± 0.001	0.009 ± 0.001	107534-96-3	3.70 ^c	32 (20°C, pH 7) ^c
Biocide	Triclosan*	0.055					3380-34-5	4.76 ^b	4.62 (25°C) ^b
Insecticide	Diazinon	0.104 ± 0.009	0.045 ± 0.007 ^S	0.044 ± 0.006	0.098 ± 0.003	0.093 ± 0.002	333-41-5	3.3 ^c	60 (20°C) ^c
Corrosion inhibitor	Benzotriazole	0.913 ± 0.019	0.568 ± 0.033 ^{SxT}	0.740 ± 0.025	0.767 ± 0.029	0.697 ± 0.050	95-14-7	1.44	5957 (25°C) ^b
Artificial sweetener	Sucralose	1.187 ± 0.020	1.233 ± 0.021	1.342 ± 0.041	1.212 ± 0.014	1.220 ± 0.014	56038-13-2	-1.00 ^a	22750 (25°C) ^a

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768 Table 2. Results from multivariate and univariate permutational analyses separately for heat wave phase and recovery phases (Recovery 1 and
 769 Recovery 2) of the experiment. Multivariate analyses include all response variables listed in this table. η^2 indicates the proportion of variance
 770 explained by each factor. Bold values indicate significant results at $\alpha=0.05$. Covariate is based on body size.

	Heat wave					Recovery 1					Recovery 2				
	df	MS	Pseudo-F	η^2	P (perm)	df	MS	Pseudo-F	η^2	P (perm)	df	MS	Pseudo-F	η^2	P (perm)
Multivariate															
Covariate	1	27.491	5.646	0.0694	0.0002	1	22.797	4.012	0.068	0.0014	1	27.048	4.957	0.078	0.0004
Chemical	1	10.271	2.110	0.0259	0.040	1	1.726	0.304	0.005	0.928	1	12.842	2.353	0.037	0.028
Temperature	1	48.706	10.003	0.1230	0.0001	1	2.254	0.397	0.007	0.877	1	1.562	0.286	0.004	0.942
Chem x Temp	1	7.640	1.569	0.0193	0.137	1	13.772	2.424	0.041	0.031	1	11.880	2.177	0.034	0.436
Residual	62	4.869		0.7623		52	5.682		0.879		54	5.457		0.847	
Respiration															
Covariate	1	7.115	17.715	0.108	0.0003	1	13.878	18.033	0.248	0.0002	1	5.451	6.268	0.094	0.015
Chemical	1	0.128	0.318	0.002	0.579	1	7.06E-02	9.18E-02	0.001	0.763	1	1.817	2.090	0.031	0.171
Temperature	1	32.145	80.038	0.487	0.0001	1	1.216	1.578	0.022	0.213	1	0.486	0.559	0.008	0.467
Chem x Temp	1	1.712	4.264	0.026	0.044	1	0.817	1.061	0.015	0.317	1	3.282	3.774	0.057	0.062
Residual	62	0.402		0.377		52	0.770		0.715		54	0.870		0.810	
Food consumption															
Covariate	1	7.223	8.177	0.103	0.006	1	3.885	4.045	0.059	0.048	1	11.704	15.246	0.170	0.0003
Chemical	1	0.228	0.258	0.003	0.613	1	6.71E-04	6.99E-04	0.002	0.698	1	2.106	2.743	0.031	0.111
Temperature	1	0.364	0.412	0.005	0.526	1	0.04	0.04	0.000	0.982	1	0.979	1.275	0.014	0.258
Chem x Temp	1	3.887	4.400	0.056	0.040	1	2.419	2.519	0.037	0.116	1	4.314	5.620	0.063	0.023
Residual	66	0.883		0.833		62	0.96		0.902		65	0.768		0.723	
Specific growth rate															
Covariate	1	0.837	0.844	0.012	0.356	1	3.108	3.480	0.047	0.067	1	6.252	6.625	0.091	0.011
Chemical	1	1.874	1.890	0.027	0.195	1	0.529	0.592	0.008	0.446	1	0.222	0.235	0.003	0.633
Temperature	1	1.677	1.691	0.024	0.217	1	0.016	0.018	0.000	0.898	1	0.246	0.261	0.004	0.617
Chem x Temp	1	0.181	0.182	0.003	0.712	1	6.973	7.807	0.106	0.005	1	0.938	0.994	0.014	0.321
Residual	66	0.991		0.935		62	0.893		0.839		65	0.944		0.889	

Number of eggs

Covariate	1	7.776	10.832	0.116	0.002	1	0.600	0.579	0.010	0.450	1	10.164	11.956	0.167	0.0009
Chemical	1	3.344	4.659	0.050	0.032	1	0.376	0.363	0.006	0.547	1	1.163	1.368	0.019	0.251
Temperature	1	9.235	12.865	0.138	0.0002	1	0.315	0.304	0.005	0.583	1	0.908	1.069	0.015	0.308
Chem x Temp	1	1.422	1.982	0.021	0.162	1	0.746	0.721	0.013	0.403	1	0.307	0.361	0.005	0.546
Residual	63	0.718		0.675		55	1.036		0.965		57	0.85		0.794	

PO-like activity

Covariate	1	2.294	2.534	0.033	0.116	1	2.936	2.906	0.044	0.096	1	0.738	0.738	0.011	0.388
Chemical	1	0.001	0.001	0.000	0.974	1	0.166	0.164	0.003	0.698	1	2.588	2.586	0.038	0.116
Temperature	1	7.238	7.996	0.105	0.003	1	0.242	0.240	0.004	0.629	1	0.01	0.01	0.000	0.922
Chem x Temp	1	0.630	0.696	0.009	0.416	1	0.008	0.007	0.000	0.932	1	0.632	0.631	0.009	0.434
Residual	65	0.905		0.853		62	1.101		0.949		65	1.0005		0.943	

Antibacterial activity

Covariate	1	5.379	5.805	0.078	0.019	1	5.209	5.889	0.079	0.018	1	6.525	7.036	0.095	0.011
Chemical	1	2.954	3.188	0.043	0.081	1	0.006	0.007	0.000	0.935	1	0.725	0.782	0.011	0.382
Temperature	1	0.404	0.436	0.006	0.515	1	2.855	3.229	0.043	0.071	1	1.431	1.543	0.021	0.218
Chem x Temp	1	0.036	0.039	0.001	0.846	1	3.096	3.501	0.047	0.067	1	0.042	0.045	0.001	0.833
Residual	65	0.927		0.873		62	0.884		0.831		65	0.927		0.874	

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774 Table 3. Results from univariate permutational analyses separately for heat wave phase and recovery phases (Recovery 1 and Recovery 2) of the
 775 experiment. “Eggs (yes/no)” is based on binomial data on whether snails reproduced or not. η^2 indicates the variance explained by each factor. Bold
 776 values indicate significant results at $\alpha=0.05$. Covariate is based on body size.

	Heat wave					Recovery 1					Recovery 2				
	df	MS	Pseudo-F	η^2	P (perm)	df	MS	Pseudo-F	η^2	P (perm)	df	MS	Pseudo-F	η^2	P (perm)
Eggs (yes/no)															
Covariate	1	0.002	0.054	0.001	0.820	1	0.255 4.24E-04	2.736	0.041	0.103	1	0.030	0.307	0.004	0.586
Chemical	1	0.016	0.396	0.006	0.542	1	0.005	0.005	0.000	0.948	1	0.065	0.654	0.009	0.423
Temperature	1	0.122	2.975	0.043	0.157	1	0.009	0.097	0.001	0.775	1	0.525 6.08E-05	5.281 6.11E-04	0.074	0.027
Chem x Temp	1	0.017	0.419	0.006	0.522	1	0.233	2.501	0.037	0.116	1	0.05	0.04	0.000	0.975
Residual	66	0.041		0.945		62	0.093		0.921		65	0.099		0.912	
Hatching %															
Covariate	1	9.275	10.494	0.138	0.001	1	0.349	0.335	0.006	0.561	1	1.337	1.293	0.022	0.262
Chemical	1	0.043	0.049	0.001	0.831	1	1.380	1.327	0.023	0.256	1	0.095	0.092	0.002	0.773
Temperature	1	0.678	0.767	0.010	0.389	1	0.023	0.022	0.000	0.889	1	0.279	0.270	0.005	0.613
Chem x Temp	1	1.321	1.495	0.020	0.232	1	0.059	0.056	0.001	0.814	1	0.335	0.324	0.005	0.579
Residual	63	0.884		0.831		55	1.04		0.969		57	1.034		0.966	
Number of hatched eggs															
Covariate	1	0.376	0.395	0.006	0.529	1	0.012	0.012	0.000	0.919	1	11.147	12.936	0.183	0.0005
Chemical	1	1.291	1.357	0.019	0.248	1	0.116	0.109	0.002	0.744	1	0.487	0.565	0.008	0.454
Temperature	1	5.390	5.666	0.080	0.018	1	0.243	0.229	0.004	0.631	1	0.207	0.241	0.003	0.619
Chem x Temp	1	0.013	0.014	0.000	0.908	1	0.409	0.386	0.007	0.532	1	0.041	0.047	0.001	0.824
Residual	63	0.951		0.894		55	1.06		0.987		57	0.862		0.805	

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FIGURE CAPTIONS

Fig. 1. Respiration rate (a), food consumption (b) and specific growth rate (c) of snails in no stress treatment (open circles, i.e. constant temperature, no MPs), with one added stressor (grey symbols: exposure to heat wave (triangles) or micropollutants (diamonds)) and two added stressors (black squares; i.e. exposure to both heat wave and micropollutants) during the heat wave phase, eight days after the heat wave (Recovery 1 -phase) and 16 days after the heat wave (Recovery 2-phase). Data are mean $\pm$ SE.

Fig. 2. Interaction effect sizes based on Cohen's d indicating whether the observed combined effects of the heat wave and micropollutants differed (synergistically or antagonistically) from the expected (additive) effects. Data presented separately for the heat wave-phase, eight days after the heat wave (Recovery 1) and 16 days after the heat wave (Recovery 2). Data are mean $\pm$ 95% CI. Positive values indicate synergistic effects, values crossing the zero-line indicate additive effects and negative values indicate antagonistic effects of heat waves and micropollutants on the studied response variables. Asterisks indicate statistically significant interactive effects on the studied traits.

Fig. 3. Number of produced eggs (a), number of offspring (b), PO-like activity (c) and antibacterial activity (d) of snails in no stress treatment (open circles), with one added stressor (grey symbols: exposure to heat wave (triangles) or micropollutants (diamonds)) or two added stressors (black squares) during the heat wave phase, eight days after the heat wave (Recovery 1 -phase) and 16 days after the heat wave (Recovery 2-phase). Data are mean $\pm$ SE.

Fig. 4. Summary of the immediate effects of exposure to the experimental heat wave, mixture of micropollutants and combination of both after the heat wave phase. Black and grey lines indicate

803 significant positive and negative treatment effects on traits, respectively. Dashed lines indicate resulting
804 imbalance in resource levels due to increased metabolic demand.

SUPPLEMENTARY MATERIAL:

Micropollutant analyses

Micropollutant (henceforth MP) concentrations in the water were assessed by collecting samples of the MP enriched water before (n=1) and after (n=8) the two day incubation on three occasions during the heat wave (in total 27 samples). Samples were collected from water incubated both with and without snails (control) from both temperatures. The samples were kept in -20 °C until analysed. Online enrichment (Huntscha *et al.*, 2012) was used to detect the compounds administered in low concentrations. 100 ml of filtered (GF/F, Whatman) sample was spiked with internal standard containing isotope labelled compounds. Metformin concentration was analysed on non-enriched samples. After centrifugation (30 min at 4 000 rpm) 1 ml of the supernatant was spiked with internal standard containing isotope labelled compounds. The chromatography was performed on a C18 Atlantis T3 column (particle size 3 µm, 3.0 x 150 mm, Waters) with precolumn using following gradient: MeOH +0.1% FA, H₂O +0.1% FA, Gradient 300ul 95/5 for 1.5 min, to 5/95 in 17 min, constant for 8 min, to 95/5 in 0.5 min, constant for 4.5 min. All samples were measured at a high resolution mass spectrometer (Q-Exactive, Thermo Scientific) in a fullscan experiment in positive and negative mode at a resolution of 70 000 and a scan range of 100-1 000 m/z followed by data dependent MS/MS experiments at a resolution of 17 500. Quantification was conducted using Tracefinder 3.2 software (Thermo Scientific). The compounds were quantified within a mass window of 5 ppm.

Impact of temperature and presence of snail on MP concentrations were analysed in separate univariate permutational analysis for each chemical with factors temperature (14°C or 23.5°C), snail (presence or absence of snail) and their interaction.

Concentration of majority of the MPs was not affected by neither temperature nor presence of snails (Table 1). Presence of snails reduced the concentration of diazinon and diclofenac significantly (Pseudo- $F_{1,22}=103.3$ $p=0.0001$ and Pseudo- $F_{1,22}=43.0$ $p=0.0001$, respectively). Both high temperature and presence of snails decreased clarithromycin concentration slightly (Pseudo- $F_{2,22}=5.77$ $p=0.012$ and Pseudo- $F_{1,22}=6.91$ $p=0.009$, respectively) while these factors had interactive impacts on valsartan and benzotriazol (Pseudo- $F_{1,22}=10.2$ $p=0.0037$ and Pseudo- $F_{1,22}=12.4$ $p=0.0011$, respectively). Post hoc comparisons revealed that valsartan concentration was significantly lower in 14°C compared to 23.5°C when incubated with snails and that benzotriazol concentration was significantly lower after incubation in 14°C with a snail compared to all the other treatment combinations.

Immune defence analyses

Snails were blot dried with paper towels and the foot was tapped to induce secretion of haemolymph (Sminia, 1981). To measure PO-like activity, 10 µl fresh haemolymph was mixed with 100 µl of phosphate buffered saline (PBS) and snap frozen, while 100 µl fresh haemolymph was snap frozen to measure antibacterial activity. The samples were stored in -80°C and analysed using L-Dopa and E-coli assays for PO-like activity and antibacterial activity, respectively, implementing methods in Leicht, Jokela & Seppälä (2013). A slight modification was made for incubation temperature for the antibacterial analysis that was adjusted to +24°C. Briefly, when L-Dopa is added to the haemolymph sample, it is oxidized by the enzyme PO causing an increase in optical density. In an E-coli assay, antibacterial enzymes in the haemolymph destroy lyophilized *E. coli* cells added to the solution - thereby decreasing optical density. The changes in optical density with time (greater change indicates greater enzymatic activity) were measured spectrophotometrically (SpectraMax 190, Molecular Devices, Sunnyvale, CA, USA). To assess

the repeatability of both PO-like activity and antibacterial activity measurements, duplicate samples from 36 and 38 snails, respectively, were collected and analysed. Repeatability of immune parameters was calculated from variance components of a one-way ANOVA with snail as a fixed factor. Repeatability was high for both PO-like ($R = 0.845$, $F_{35,36} = 11.92$, $p < 0.001$) and antibacterial ($R = 0.621$, $F_{37,38} = 4.273$, $p < 0.001$) activity.

56 Table S1. Results from univariate permutational analyses based on technical control vs. control data separately for heat wave phase and
 57 recovery phases (Recovery 1 and Recovery 2) of the experiment. Bold values indicate significant results at $\alpha=0.05$. Covariate is based on
 58 body size.

	Heat wave				Recovery 1				Recovery 2			
	df	MS	Pseudo-F	P (perm)	df	MS	Pseudo-F	P (perm)	df	MS	Pseudo-F	P (perm)
Respiration												
Covariate	1	30.183	77.079	0.0001	1	0.59653	0.60051	0.4336	1	0.18078	0.17492	0.6693
Chemical	1	0.332	0.84783	0.3553	1	6.30E-02	6.35E-02	0.8026	1	0.26862	0.25991	0.6223
Temperature	1	11.113	28.38	0.0001	1	2.5142	2.531	0.1173	1	7.70E-02	7.45E-02	0.7825
Chem x Temp	1	9.39E-02	0.23984	0.6293	1	1.2174	1.2255	0.2733	1	1.396	1.3507	0.2399
Residual	62	0.39158			59	0.99337			62	1.0335		
Food consumption												
Covariate	1	14.067	17.695	0.0002	1	0.38967	0.37666	0.5378	1	1.444	1.5832	0.2149
Chemical	1	6.49E-02	8.16E-02	0.7694	1	1.2946	1.2514	0.2735	1	6.4199	7.0385	0.0099
Temperature	1	2.9768	3.7447	0.0575	1	0.17538	0.16952	0.672	1	6.31E-03	6.92E-03	0.9328
Chem x Temp	1	1.41E-02	1.77E-02	0.891	1	3.22E-02	3.11E-02	0.8622	1	1.7544	1.9234	0.1647
Residual	64	0.79496			61	1.0346			64	0.91211		
Specific growth rate												
Covariate	1	7.85E-02	7.58E-02	0.7838	1	0.22384	0.24166	0.6218	1	1.1974	1.1929	0.2815
Chemical	1	1.3179	1.2726	0.3147	1	2.60E-03	2.81E-03	0.9578	1	0.70382	0.70119	0.4057
Temperature	1	0.28622	0.27638	0.732	1	2.0436	2.2063	0.149	1	2.11E-02	2.10E-02	0.8862
Chem x Temp	1	3.93E-02	3.80E-02	0.918	1	6.0803	6.5644	0.0113	1	1.8376	1.8307	0.1742
Residual	64	1.0356			59	0.92626			64	1.0038		
Number of eggs												
Covariate	1	2.8817	4.2911	0.044	1	2.05	2.2166	0.1446	1	7.54E-02	7.10E-02	0.7906
Chemical	1	0.2543	0.37867	0.5465	1	5.60E-03	6.05E-03	0.9407	1	0.45565	0.42876	0.5137
Temperature	1	19.758	29.421	0.0001	1	3.3946	3.6704	0.06	1	5.32E-02	5.01E-02	0.8263
Chem x Temp	1	1.1406	1.6984	0.1982	1	2.5317	2.7374	0.1035	1	2.86E-02	2.69E-02	0.8729
Residual	61	0.67156			53	0.92487			54	1.0627		

Number of hatched eggs												
Covariate	1	0.79728	0.90513	0.3427	1	0.21561	0.22228	0.6448	1	2.62E-02	2.47E-02	0.877
Chemical	1	1.6626	1.8876	0.176	1	7.60E-03	7.83E-03	0.9286	1	5.16E-02	4.87E-02	0.8201
Temperature	1	8.3896	9.5246	0.0024	1	2.8252	2.9128	0.0898	1	0.40811	0.38453	0.5317
Chem x Temp	1	0.41926	0.47598	0.4929	1	2.5441	2.623	0.1093	1	0.20224	0.19055	0.6653
Residual	61	0.88084			53	0.96995			54	1.0613		
Hatching %												
Covariate	1	12.633	17.032	0.0003	1	0.43054	0.40592	0.524	1	5.81E-04	5.58E-04	0.9803
Chemical	1	0.98395	1.3266	0.2527	1	7.04E-02	6.64E-02	0.8114	1	0.27132	0.26029	0.6121
Temperature	1	5.9202	7.9816	0.0059	1	0.26858	0.25322	0.6369	1	0.92772	0.89001	0.3592
Chem x Temp	1	0.21718	0.2928	0.5851	1	1.50E-02	1.42E-02	0.9103	1	0.51227	0.49144	0.5054
Residual	61	0.74173			53	1.0607			54	1.0424		
PO-like activity												
Covariate	1	2.967	4.1531	0.0429	1	0.10089	9.71E-02	0.7551	1	0.63622	0.61578	0.4374
Chemical	1	0.54426	0.76183	0.3783	1	1.242	1.1948	0.2765	1	1.1763	1.1386	0.3323
Temperature	1	18.481	25.869	0.0001	1	0.23386	0.22496	0.6381	1	4.51E-02	4.37E-02	0.8975
Chem x Temp	1	0.28549	0.39961	0.5323	1	4.91E-02	4.73E-02	0.8295	1	1.83E-02	1.77E-02	0.9285
Residual	64	0.71441			60	1.0396			64	1.0332		
Antibacterial activity												
Covariate	1	6.7489	8.3235	0.0047	1	0.64819	0.64445	0.4301	1	1.80E-03	1.75E-03	0.9664
Chemical	1	2.4838	3.0633	0.0889	1	1.7466	1.7366	0.1992	1	0.46252	0.44856	0.5031
Temperature	1	5.0519	6.2305	0.016	1	5.86E-02	5.83E-02	0.8093	1	1.506	1.4605	0.2309
Chem x Temp	1	1.822	2.2471	0.1378	1	1.1991	1.1922	0.2866	1	3.75E-02	3.64E-02	0.8471
Residual	64	0.81083			60	1.0058			64	1.0311		

Figure S1. (a) Schematic representation of the experimental design and the sampling events. The grey boxes illustrate the time when snails in the respective treatment were sampled (after the heat wave phase, eight or 16 days of recovery). The individuals experienced the same chemical treatment throughout the experiment and, thus, the recovery only reflects to the heat wave. Each treatment combination for each sampling event was replicated 18 times. (b) Sampling during the eight days experimental phase during the heat wave, recovery 1 and recovery 2 phases. Snail length was measured at the beginning and at the end of the time period, respiration during the second last day, food consumption during the last 24-27 hours, while PO-like and antibacterial activity as well as the number of eggs at the end of the time period. Water changes and mortality checks are illustrated by grey arrows. These were also conducted at the beginning and at the end of the eight-days period.

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