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Synergistic interaction between effects of phenanthrene and dynamic heat stress cycles in a soil arthropod

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Abstract

Climatic stressors and chemicals should not be treated as isolated problems since they often occur simultaneously, and their combined effects must be evaluated including their possible interactive effects. In the present study we subjected springtails (*Folsomia candida*) to combined exposure to phenanthrene and dynamic heat cycles in a full factorial experiment. In a microcosm experiment, we studied the population growth of springtails subjected to a range of sub-lethal concentrations of phenanthrene. During the 28-day experiment we further subjected microcosms to varying numbers of repeated dynamic heat cycles (0-5 cycles) simulating repeated heat waves. We found a synergistic interaction between the effects of phenanthrene and the number of heat waves on both body mass of adults and juvenile production of *F. candida* showing that the negative effects of phenanthrene were intensified when animals were heat stressed, and/or vice versa. This interaction was not related to internal concentrations of phenanthrene in adult springtails, nor was it due to altered degradation of phenanthrene in soil. We argue that both phenanthrene (by its partitioning into membrane bilayers) and heat have detrimental effects on the physical conditions of cellular membranes in a dose-dependent manner, which, under extreme circumstances, can increase membrane fluidity to a level which is sub-optimal for normal membrane functioning. We discuss the possibility that the synergistic interactions subsequently reduce life-history parameters such as growth and reproduction.

Key words: multiple stressors, temperature, membrane fluidity, reproduction, survival

Capsule: Realistic heat stress aggravates the effects of phenanthrene on soil arthropods

Introduction

The Intergovernmental Panel on Climate Change (IPCC) reports that we are facing increasing global temperatures within the next century and that the frequency and intensity of extreme heat events are going to increase (IPCC, 2014). Climate change, and especially extreme weather events, are becoming an urgent problem because they have many far-reaching consequences for species and ecosystems on Earth (Easterling et al., 2000; Parmesan and Yohe, 2003; Walther et al., 2002). This is particularly important for ectothermic organisms such as arthropods because their body temperature is dictated by habitat temperature. Thus, extremely high and low temperatures can be damaging to the individual, and the thermal tolerance of species often sets the limits for the geographic distribution of populations (Huey and Kingsolver, 1989; Kellermann et al., 2012). Environmental change such as warming of a habitat may result in physiological constraints on individuals, which will have impacts on fitness and ultimately cause changes in community composition of the particular habitat (Sinclair et al., 2016). The further implications of species losses or changed composition of communities due to climate change may be reductions in the ecosystem services such as degradation of organic matter and water infiltration delivered by species (Blouin et al., 2013; de Vries et al., 2013). In addition, due to industrial and agricultural activities, a range of potentially toxic chemicals are released into the environment putting species, communities and ecosystems under pressure (Köhler and Triebkorn, 2013). The consequences of environmental contaminants may be modified by climate change because climate can alter not only the fate and distribution, but also biological effects, of contaminants (Hooper et al., 2013; Noyes et al., 2009). Since organisms face environmental stress from climate change as well as contamination, the combined effects on ecological receptors is currently an important area of research to explore.

Many studies show that contaminants influence the tolerance of natural stressors such as heat, drought or cold and vice versa (Holmstrup et al., 2010). A common explanation for this is that cellular

or physiological defence mechanisms towards any type of stress has costs in terms of energy leaving less energy resources to combat negative effects of an additional stressor (Calow, 1991). Examples of defence mechanisms against contaminants include cellular detoxification of contaminants by induction of both phase I (e.g. cytochrome P₄₅₀, carboxyl esterase) and phase II (e.g. glutathione-S-transferase) enzymes (Walker et al., 2001). Heat shock proteins (Hsp), and in particular the inducible Hsp70, is important for survival of thermal stress. However, as on detoxification systems, it often comes with a cost for individual growth and reproduction (Hoffmann et al., 2003).

Lipophilic contaminants such as Polycyclic Aromatic Hydrocarbons (PAH) are widespread in soils as a result of sewage sludge deposition and burning of organic matter and fossil fuels (Heywood et al., 2006; Petersen et al., 2003). Polycyclic aromatic hydrocarbons can partition into cellular membranes because of their lipophilic properties, and thereby cause non-specific “baseline toxicity” due to altered fluidity of the membrane and the consequences this has for functioning of membrane proteins (Holmstrup et al., 2014a; Van Wezel et al., 1996). Effects of lipophilic contaminants are therefore likely to interfere with thermal stress because tolerance of extreme temperatures depends on the organism’s ability to compensate for the altered fluidity associated with heat or cold (Bowler, 2018; Cossins and Raynard, 1987; Hazel, 1995).

Gaps in knowledge exist on the combined effects of heat and contaminants. Most multiple stressor studies of terrestrial organisms involving combinations of heat stress and contaminants have mainly been concerned with survival as an endpoint (Holmstrup et al., 2010; Laskowski et al., 2010), whereas chronic (sub-lethal) effects expressed through reproduction or physiological parameters are relatively scarce (but see Janssens et al., 2014; Jegede et al., 2017; Menezes-Oliveira et al., 2013; Sniegula et al., 2017). Further, most studies have used constant temperature exposures, whereas organisms in nature are exposed to fluctuating temperatures with heat bouts alternating with less severe

temperatures allowing for recovery and repair of damages resulting from extreme temperatures. These considerations encouraged us to study the combined effects of a common lipophilic contaminant (phenanthrene; PHE) and repeated heat exposure using soil microcosms with the springtail, *Folsomia candida* Willem. The effects of PHE on survival and reproduction of *F. candida* have been studied by several authors, but virtually nothing is known about the potential interactions between effects of heat stress and PHE (Crouau et al., 1999; Droge et al., 2006; Gainer et al., 2019; Nota et al., 2009; Paumen et al., 2008). Springtails (Collembola) are widespread soil microarthropods and represent organisms with importance for soil functions such as decomposition of dead organic matter and nutrient cycling (Hopkin, 1997).

We performed a full factorial 28-day experiment combining repeated, ramping heat cycles (0, 1, 2, 3, 4 or 5 heat cycles, respectively) with a range of sub-lethal concentrations of PHE (0-80 mg kg⁻¹ dry soil) and quantified survival, adult body mass and production of juveniles. Exposure to acute high temperature increases the fluidity of cellular membranes (Hazel, 1995) and partitioning of PHE into membranes has the same effect (Holmstrup et al., 2014a). If the combined effect of these two stressors cause membranes to reach a detrimental upper threshold for fluidity we should observe combined effects that are higher than expected from the effects of each stressor alone. We therefore hypothesized that effects of repeated heat cycles and PHE exposure will interact synergistically with respect to springtail performance.

Materials and methods

Test animals

Folsomia candida (Collembola, Isotomidae) originated from a laboratory culture kept at 20 °C (±1 °C)

and in a 12:12 h light-dark cycle on Petri dishes with moistened plaster of Paris mixed with charcoal (8:1 w/w). The springtails were fed dried baker's yeast and kept at a density of ca. 120 animals in each Petri dish. The springtails were not age-synchronized, however, in order to minimize variation in individual responses only medium sized adults of roughly the same size were used (approximately 0.15 mg fresh weight). For all experiments, we selected adults of similar size from the cultures and distributed them at random to the various treatments.

Test soil

An organically grown (i.e. not treated with pesticides or pharmaceuticals of any kind for more than 12 years) field soil was collected at Askov, Denmark. The soil was dried at 80 °C for 24 h and sieved through a 2 mm mesh prior to use. The Askov soil is characterized as a sandy loam with the following particle distribution: coarse sand (200-2000 µm) 38.4 %, fine sand (63-200 µm) 23.6 %, coarse silt (20-63 µm) 10.0 %, fine silt (2-20 µm) 12.3 %, and clay (<2 µm) 13.0 %. The total organic carbon content was 1.6 % (determined by loss-on-ignition), and soil pH_{water} was 6.2 (deionized water). Bulk density, water-holding capacity and the total cation exchange capacity (CEC) were 1.135 g/cm³, 36 % (w/w) and 8.14 meq (100 g)⁻¹, respectively.

PHE contaminated soil

The soil was spiked with PHE (Sigma Aldrich, CAS #85-01-8, 98% purity) using acetone as a carrier solvent. PHE was dissolved in acetone (J.T. Barker, HPLC quality) to obtain the following concentrations (0 (acetone control), 9.9, 20.3, 30.3, 40.3, 58.9 and 80.4 mg kg⁻¹ dry soil). These concentrations were deemed appropriate for detection of sub-lethal effects according to a previous study (Holmstrup et al., 2014a). The spiked soil was thoroughly mixed, and left overnight under a fume

hood to allow acetone to evaporate. The soil was stirred once again to ensure that complete evaporation of acetone occurred. Subsequently, water content of the soil was adjusted to 50% of the water holding capacity (180 mL kg⁻¹ dry soil) using deionized water. The moist soil was thoroughly mixed.

Establishing sublethal heat treatments

We wanted to apply an environmentally realistic and relatively mild heat treatment that did not cause acute lethality. Therefore, we performed a range-finding experiment in which we subjected springtails to various high temperatures. Ten springtails were introduced to 50 mL glass beakers containing 20 g moist uncontaminated soil and the vials were closed with a perforated plastic lid allowing gas exchange. The beakers were then exposed to gradual (i.e. constant slope) heating from 20 to 40 °C using a warming rate of 4 °C h⁻¹. The gradual heating was achieved using a programmable temperature cabinet precise within ±0.1 °C (Binder WTB Labortechnik, Tuttlingen, Germany). The actual (measured) temperature at the soil surface and in soil was the same as the programmed air temperature of the cabinet (Supplementary Information Fig S1). Five replicates were sampled when the temperature reached 24, 28, 32, 36, 38, 38.5, 39, 39.5 and 40 °C, respectively. The soil of each beaker was immediately spread out in separate trays at room temperature and the springtails were collected using an aspirator and transferred to a Petri dish containing water-saturated charcoal/plaster of Paris mixture. Survival was assessed after recovery at 20 °C for 24 h. Only springtails that were able to walk in a coordinated manner upon gentle tactile stimulation were scored as survivors. Subsequently, the animals of the five replicates of each treatment were combined and 12 surviving springtails were collected at random from temperature treatments where survival was sufficiently high (24, 28, 32, 36 and 38 °C) and transferred singly to separate Petri dishes with a few grains of dried yeast. During the following week, we inspected the springtails daily and noted the time to first egg-laying and the number of eggs

produced.

Toxicity of PHE

Based on pilot studies we chose a range of seven sublethal PHE concentrations from 0 to 80.4 mg kg⁻¹ dry soil and performed a 4-week reproduction test following previous descriptions (Sørensen and Holmstrup, 2005). In brief, ten randomly picked adult springtails were added to each 50 mL glass beaker containing 20 g moist, freshly spiked soil. About 2 mg dried baker's yeast was added to the surface of the soil as their food, and the vials were closed with a perforated plastic lid. The glass beakers were kept at 20 ± 1 °C with a 12:12 h light:dark regime. Once a week, all the vials were weighed and deionized water was added to compensate for evaporation losses. Further, each beaker was replenished with 2 mg dried baker's yeast. After 28 days, test soil of each beaker containing the springtails (adults and hatched juveniles) was transferred to a plastic cylinder (diameter 6 cm; height 5.5 cm) which was closed in one end by a 0.5 mm mesh. The cylinders were placed in a high gradient MacFadyen-type extraction apparatus, where the temperature in the upper compartment (at soil surface) was ramped from 25 to 46 °C during 7 h while the temperature at the lower compartment remained constant at 5 °C. The extracted springtails dropped into a beaker with water. After extraction for 7 h, the soil of each cylinder was transferred to a 100 mL beaker containing 80 mL deionized water and stirred gently allowing the remaining springtails to be collected by floatation. Three drops of blue ink were added to increase the contrast between the white animals and the water on which they were floating. The floating animals were photographed for later counting using Image J software (Rueden et al., 2017). Immediately thereafter the originally added adults, and juveniles produced during the test (the original adults added to beakers were easily separated by their large size), were transferred to separate Eppendorf vials and stored at -80 °C until further analysis. Adults of each replicate were

counted and their combined fresh mass determined to the nearest μg (Sartorius AG, Goettingen, Germany). Average adult fresh weight of each replicate was calculated.

Combined effect of heat and PHE on adult survival, body mass and juvenile production

We tested for combined effects of PHE and recurring heat exposure (constant warming rate ramping from 20 to 36 °C over 4 hours and then immediately back to 20 °C) in a full factorial test design using six replicates per treatment combination. The chosen heat treatment was based on results shown in Fig. 1 and was anticipated to have no or very moderate acute lethal effects, but negative effects on egg production (Fig. 2). To this end we applied seven concentrations of PHE (including uncontaminated control) combined with 0 (heat control), 1, 2, 3, 4 or 5 heat cycles, respectively. The first heat cycle was applied on the first day of the experiment and followed by 4 days of recovery at 20 °C before the next heat cycle was applied on the fifth day, and so forth. The complete design of the full factorial experiment is outlined in Fig S2. After 28 days the experiment was terminated, and springtails were extracted as described (see previous section “Toxicity of PHE”), counted and frozen at -80 °C for later analysis as described above.

Degradation of PHE in soil

To verify nominal concentrations and degradation of PHE in soil over the 28-day experiment we used four replicates consisting of 5 g moist soil in 10 mL glass vials, but otherwise treated in the same manner as described in the previous section. These samples were taken at the end of the 28-day test period.

Determination of PHE in springtail tissue and soil

Phenanthrene concentrations of the animal tissues were determined according to a method described in details by Holmstrup et al. (2014a). Briefly, the combined fresh weight of adult springtails of each replicate was determined using a Sartorius Micro SC 2 balance accurate to $\pm 1 \mu\text{g}$ (Sartorius AG, Goettingen, Germany). The samples were transferred to 1.5 mL brown glass vials and 500 μL of acetonitrile was added. The samples were placed on ice, sonicated for 90 min and then kept at room temperature for 24 h, frozen at -18°C for 24 h and finally kept at room temperature for 24 h. The samples were again sonicated for 90 min on ice and were then transferred to Eppendorf vials. After brief centrifugation (3 min at 2,620 g), the supernatant was transferred to autosampler vials and stored at -80°C until analysis by GC-MS. PHE standards including blanks were run in parallel and subjected to the same extraction procedure. A Shimadzu GCMS-QP2010 with an autosampler was used to perform the analysis. Recovery was tested by spiking uncontaminated animal material with known amounts of PHE. The recovery of PHE was on average 95%, however, we have not corrected for this when reporting internal concentrations.

Phenanthrene in representative soil samples (1 g fresh weight) were extracted with 4 mL acetonitrile by shaking at 200 rpm for 24 h followed by centrifugation at 3,000 rpm for 5 min. The supernatant was transferred to autosampler vials and analysed as described above.

Statistical modelling and analysis

Adult survival of heat stress was analyzed by a two-parametric log-logistic dose response curve model using “drc” package in R (ver. 3.4.1). Survival data were binomial distributed. The effect of high temperature on egg production, the internal content of PHE in springtail tissue, and the PHE

degradation in soil was analyzed using generalized linear models in the package “lme4” in R (ver. 3.4.1). For egg production the model was fitted using the `glmer()` function, with Poisson as family type, log as the link function, and animal replicate as random factor. The models for PHE content in springtail tissue, and for PHE degradation in soil, were fitted using the `glm()` function, with gamma as family type and inverse as the link function.

In the analysis of data from the full factorial experiment, the effects of PHE and heat, as well as any possible interaction between PHE and heat on survival were modelled using a general sigmoid dose-response function,

$$f(x; b, x_0) = \frac{1 + \exp(-b x_0)}{1 + \exp(b(x - x_0))} \quad x \geq 0, b \geq 0 \quad (1),$$

where $f(x)$ is the expected effect of a single stress factor x , x_0 is the point of inflexion, and b is the shape parameter of the function, i.e. the slope at the point of inflexion. If $b = 0$, then $f(x) = 1$.

The modes of action of PHE and heat were assumed to be independent, and therefore the combined effect of these two stressors was estimated using a multiplicative model (Bindesbøl et al., 2005). Thus, the combined effect of the stressors and the interaction effect on the expected probability of survival were modelled as:

$$p(c, t) = (1 - \lambda) f(c; b_c, x_{0,c}) f(t; b_t, x_{0,t}) f(c \cdot t; b_{ct}, x_{0,ct}) \quad (2),$$

where c is the level of PHE, t is the level of heat (i.e. number of heat cycles), and $\lambda \in [0, 1]$ is the

residual or control mortality. Note that the interaction effect of combining PHE and heat is assumed to be a dose-response function of the level of PHE multiplied by the level of heat. A more detailed description of the model is described elsewhere (Damgaard et al., 2002; Højer et al., 2001).

Equation (2) was fitted to survival data assuming the number of survivors were binomially distributed with an expected probability calculated by Equation (2) (Damgaard et al., 2002). The log-likelihood function of the regression model was maximised using the *NMaximize* routine in *Mathematica* (Wolfram, 2016) with the described constraints on the parameters. The fit of the model was checked by comparing the observed and predicted values, as well as examining the distribution of the residuals.

The effect of PHE and heat on the observed adult biomass and juvenile production, respectively, was modelled in a similar way:

$$g(c, t) = \lambda f(c; b_c, x_{0,c})f(t; b_t, x_{0,t})f(c \cdot t; b_{ct}, x_{0,ct}) \quad (3),$$

where c is the level of PHE, t is the level of heat, and $\lambda > 0$ is the residual or control adult biomass or juvenile production, respectively.

Equation (3) was fitted to the adult biomass and juvenile production data assuming the data were either Poisson or Normal distributed, respectively. The log-likelihood function of the regression model was maximized using the *NMaximize* routine in *Mathematica* (Wolfram, 2016) with the described constraints on the parameters. The fit of the model was checked by comparing the observed and predicted values, as well as by examining the distribution of the residuals.

The hypotheses of no synergistic interactions were tested using Equation (2) or (3),

respectively, with $b_{ct} = 0$, in a likelihood ratio test with one degree of freedom. The hypothesis of no effect of c was tested using Equation (2) or (3), respectively, with $b_t = 0$ and $b_{ct} = 0$, in a likelihood ratio test with two degrees of freedom. Likewise, the hypothesis of no effect of t was tested using Equation (2) or (3), respectively, with $b_c = 0$ and $b_{ct} = 0$, in a likelihood ratio test with two degrees of freedom.

The EC_{50} values of the level of PHE and heat were calculated by $\log(2 + \exp(b x_0)) / b$ (Friis et al., 2004). The 95% credibility intervals of the EC_{50} values were calculated from the Bayesian joint posterior distribution of the parameters, which were calculated using Markov chain Monte Carlo (MCMC) methodology (Metropolis-Hastings algorithm) with a multinomial candidate distribution (100,000 iterations with a burn-in period of 1000), and where the prior distribution of the parameters was assumed to be uniformly distributed within their domain. The sampling procedure was checked by visual inspections of the sampling chains. The marginal posterior distributions of all parameters in the three models are summarized in the Supporting Information.

Results

Effect of high temperature on survival and egg production

In uncontaminated soil, adult springtails survived high temperatures up to 36 °C, but survival declined rapidly at higher temperature (Fig. 1). The temperature causing 50% lethality (LT_{50}) under these gradually increasing temperature conditions was 38.9 °C. Egg production of adults recovering from a single gradual heating bout to 36 °C was considerably lower than in control adults ($P = 0.026$, Fig. 2). Thus, 50% of control adults produced eggs within the first 3 days, whereas fewer heat exposed adults

produced eggs and did so later in the recovery period. After 4 days, the cumulative number of eggs in controls was on average 93 adult⁻¹ and significantly higher than in springtails exposed to heat, where the cumulative egg production was only 14 eggs adult⁻¹ ($P < 0.001$). Egg production of adults exposed to 28 °C was also significantly lower than controls ($P < 0.05$; Fig S3).

Effect of combined heat and PHE on adult survival and body mass

Survival of adult springtails in the full factorial experiment are shown in Fig. 3A. Overall, PHE had no significant effect on adult survival ($P = 0.99$) whereas multiple heat cycles caused a significantly reduced survival from about 95% residual survival in temperature controls (at all PHE concentrations) to about 75% survival after 5 heat cycles ($P < 0.001$). The model indicated no significant interaction between effects of PHE and heat cycles on survival ($P = 0.97$). Bayesian posterior distributions of model parameters are shown in Table S1A. Comparisons between observed survival and model expectations of the full factorial experiment are shown in Table S1B.

Average body mass of adults was significantly lower at the highest concentrations of PHE than in control soil ($P < 0.001$; Fig. 3B). An increasing number of heat cycles also had an overall negative effect on adult body mass ($P < 0.001$). Comparison between observed body mass and corresponding model estimates of the full factorial experiment are shown in Table S2B. A likelihood ratio test did not show a significant interaction between internal body concentration of PHE and the number of heat cycles ($P = 0.18$). However, inspection of Fig. 3B showed that the effect of heat cycles was only occurring at the highest concentrations of PHE (60-80 mg kg⁻¹ dry soil) suggesting that an interaction was indeed presented in the data. As a supplementary statistical analysis, we therefore performed a Bayesian statistics-based test for interactions between PHE and number of heat cycles. This test indicated a statistically significant, synergistic interaction (Table S2A; $b_{ct} > 0$).

Effect of combined heat and PHE on juvenile production

The number of juveniles was significantly reduced by PHE ($P < 0.001$) and heat cycles ($P < 0.001$), and there was a significant synergistic interaction between the effects of the two main factors ($P < 0.001$; Fig. 4). Bayesian posterior distributions of model parameters are shown in Table S3A and comparison between observed number of juveniles and corresponding model expectations of the full factorial experiment are shown in Table S3B. The strong significance of the interactive component of the multiplicative model shows the presence of an exacerbated effect on juvenile production in the experiment resulting from synergistic interaction mechanism between PHE exposure and the number of heat cycles (Bliss, 1939). Using the statistical model it is possible to calculate the effect of heat cycles in an environment contaminated by PHE and *vice versa*. This result is presented graphically in Fig. 5, which shows the number of dynamic heat cycles causing 50% reduction of juvenile production (EDHC₅₀) in a PHE polluted environment. Thus, EDHC₅₀ decreased from 3.8 cycles without PHE pollution to only 2.3 cycles at the highest PHE concentration in our experiment. Conversely, EC₅₀ of PHE decreased from 70 mg kg⁻¹ dry soil at 20 °C for 28 days to 39 mg PHE kg⁻¹ dry soil if springtails had been subjected to 5 consecutive dynamic heat cycles during the experiment.

PHE in soil and springtail tissue

At the end of the experiment (28 days) the concentration of PHE had decreased in soil (overall effect; $P < 0.05$). The relative degradation rate ranged from 8% to 20% indicating PHE concentrations had remained almost constant during the experiment (Table S4). The relative degradation of PHE was not significantly influenced by the number of heat cycles, nor by the initial PHE concentration, and no interaction between the main factors was seen (Table S4).

The content of PHE in adult tissue increased significantly with increasing nominal concentration in the soil ($P < 0.001$), but we found no significant effect of heat cycles (Fig. 6; Table S5). Linear regression of PHE in animal tissue and PHE in soil (combining all heat cycle treatments) showed that PHE in *F. candida* adult tissue can serve as an indicator for soil PHE pollution ($R^2 = 0.89$, $P < 0.01$; data not shown).

Discussion

There is no doubt that temperature extremes increase due to climate change, and that heat waves already have become stronger, of longer duration and occurring with higher frequency (EEA, 2017). This warrants the importance of considering climatic factors and extreme weather events when assessing the risk of chemicals to organisms, communities and ecosystems. In this study, we demonstrated the synergistic effects in juvenile production and adult body size of combined stress of heat and contaminants to a well-studied soil invertebrate species, *F. candida*. In the following, we will discuss the mechanisms underlying these effects and interactions, and put our results in a risk assessment perspective.

Effects of phenanthrene

Due to their lipophilic properties, PHE and other PAHs will be readily taken up from soil and partition into cuticular lipids, organismal storage of lipids and cellular membranes of springtails (Reichenberg and Mayer, 2006; Schmidt et al., 2013b). Studies of uptake kinetics have shown that the steady state for PHE in *F. candida* is reached within 7-14 days depending on the experimental conditions confirming that steady state conditions were achieved in our 28-day study (Schmidt et al., 2013b). At this point,

springtails contained 0.4-0.8 $\mu\text{g PHE mg}^{-1}$ fresh weight when exposed to soil having concentrations at or above 40 mg kg^{-1} dry soil. Assuming that lipid content of *F. candida* is 0.09 mg mg^{-1} fresh weight we estimated that this corresponded to 30-60 mmol PHE kg^{-1} lipid in congruence with a previous study (Holmstrup et al., 2014a). Phenanthrene and other lipophilic compounds are known for their interference with the physical properties of membranes (narcotic (lethal) effects) which start to occur at 40–160 mmol kg^{-1} lipid (Schmidt et al., 2013a; Van Wezel et al., 1996). In correspondence with this notion, we observed no lethal effects at the concentrations used here, but substantial impacts on juvenile production.

The detoxification of PHE involves solubilization *via* bio-transformations (phase I and II enzymes) into metabolites containing hydroxyl groups, and subsequent conjugation reactions facilitating excreability (Walker et al., 2001). The transcription of genes coding for enzymes involved in this type of detoxification is increased upon exposure to PHE in *F. candida* (Holmstrup et al., 2014b; Nota et al., 2009). In addition to direct toxicity (narcotic effects) of PHE and the resulting cellular perturbations, these detoxification processes generally consume energy that will reduce the scope for growth and reproduction in organisms (Maltby, 1999; Sibly and Calow, 1989). In line with this, our results clearly show that the body mass of adults and the number of juveniles diminished as concentration of PHE increased. Our estimate of EC_{50} for *F. candida* juvenile production at control temperature was ca. 70 (95% credibility interval 68-72) mg PHE kg^{-1} dry soil, which is in fairly good agreement with previous studies reporting EC_{50} in other soil types between 28 and 46 mg PHE kg^{-1} dry soil (Droge et al., 2006; Nota et al., 2009; Paumen et al., 2008).

Effects of high temperatures

We subjected springtails to a dynamic heat treatment (ramping from 20 to 36 °C during 4 hours) that

caused only marginal lethality (ca. 5%) after one cycle (Fig 1). This effect was similar after each additional heat bout adding up to ca. 25% lethality of adults in microcosms subjected to five consecutive heat cycles (Fig 3A). Our choice of heat treatment can of course be criticised for lack of realism in a temperate climate, but is not far from recorded soil temperatures at lower latitudes (Hu and Feng, 2003).

Exposure to temperatures above the normal physiological range has detrimental cellular effects in ectothermic organisms such as arthropods (Hochachka and Somero, 2002). One obvious effect is that fluidity of cellular membranes will increase, potentially to a level where the phospholipid bilayer locally may transition from the physiologically functional liquid-crystalline state into the inverted hexagonal state with catastrophic consequences for integral proteins and membrane permeability (Hazel, 1995; Hochachka and Somero, 2002). Bowler (2018) has proposed that membrane perturbation is the first event taking place during exposure to acute heat leading to a series of subsequent detrimental cellular reactions including leakage of cellular K^+ to hemolymph and the consequent dissipation of transmembrane ion gradients (O'Sullivan et al., 2017). Following this, ATP-generation is disrupted leading to drastic changes in cellular metabolism, which can be observed as a perturbation of homeostasis with regard to metabolite composition (Malmendal et al., 2006; Waagner et al., 2010). Although we do not have these data in the present study, such effects are likely to have occurred.

High temperature *per se* can cause denaturation of proteins, and above a certain temperature threshold, much of the normal protein synthesis is shut down at the expense of protective transcription and synthesis of heat shock proteins (Lindquist, 1986). Among heat shock proteins (Hsp) expression of especially Hsp70 is well known as a stress response in arthropods including *F. candida* (de Boer et al., 2010; Hoffmann et al., 2003; Slotsbo et al., 2009; Waagner et al., 2010). In simple terms, expression of Hsp70 (and a suite of other chaperoning proteins) allows repair and reconstitution of heat-denatured

proteins, however this damage control often comes at a cost to reproductive output (Ananthan et al., 1986; Hoffmann et al., 2003; Sørensen et al., 2003). For example, Hsp70 and heat hardening can be induced during exposure to moderately high temperature in *Drosophila* which improves survival of subsequent extreme heat exposure, but is associated with decreased reproduction (Krebs and Loeschcke, 1994). Heat hardening through induction of Hsp70 at mild heat exposure takes place in *F. candida* and other springtails and can persist for days after (Bahrndorff et al., 2009; Waagner et al., 2010). Therefore, potential heat hardening during gradual heating to 36 °C in our experiment caused a delay in egg laying of *F. candida* recovering at 20 °C (Fig. 2B). This was also evident in the full factorial experiment, where each additional heat bout had costs in terms of reduced production of juveniles.

Interactions between PHE and heat

The short intermittent heat bouts had no significant influence on degradation of PHE in the test soil, and relative degradation was limited (<20%) meaning that exposure conditions for springtails were relatively stable during the experiment. Furthermore, we did not detect any effect of heat treatments on the concentration of PHE in springtail tissues (Fig 6). Increasing temperature has an effect on both uptake and elimination of toxicants due to increased diffusion and metabolic rates, but it is difficult to predict in which direction tissue concentrations will go at elevated temperatures (Hooper et al., 2013). It is possible that both the assimilation and elimination rates increase to a similar extent, thus cancelling out the temperature effect on internal PHE concentration. This should potentially have different consequences since increased elimination and metabolism of PHE at elevated temperature must bear some energetic costs that might contribute to the synergistic interaction between PHE and heat. In our experiment, the high temperature events were of short duration (the temperature was above control

temperature for less than 3% of the total duration of the experiment), and this could be the main reason why tissue concentrations of PHE seemed unaffected by heat exposure (Fig 6). These considerations lead us to conclude that the observed synergistic interactions cannot be explained by toxicodynamic mechanisms, but rather are related to molecular and physiological phenomena.

Numerous studies have shown that temperature-survival curves for arthropods are highly skewed with moderate slopes at the low temperature end, and with steep slopes at the high temperature side as also seen in our study (Fig 1) (Deutsch et al., 2008; Huey et al., 2012). The steep negative slope at high temperatures may indicate that membrane fluidity of *F. candida* reaches a critical threshold slightly above 36 °C (under the present experimental conditions) at which point a phase transition of cell membranes occurs from the functional liquid-crystalline to the detrimental inverted hexagonal state (Hazel, 1995; Hazel and Williams, 1990; Hochachka and Somero, 2002). As argued above, PHE also increases the fluidity of membranes, and we may speculate that membrane disintegration and malfunctioning at high ambient temperature becomes more likely in springtails if the load of PHE in membranes is close to the levels where narcotic effects are reached (Hazel and Williams, 1990; Holmstrup et al., 2014a). In conclusion, our results suggest that the synergistic interaction is resulting from both high temperature and PHE partitioning bringing membrane perturbation beyond a critical level.

Perspectives

During the last twenty years there has been increased focus on interactions between effects of contaminants in the environment and high temperature. In order to improve the current risk assessment of chemicals in the environment there is a need for ecotoxicological studies that include realistic climatic conditions, but also extreme weather events. With our study, we have generated new

information by showing that repeated heat waves have drastic effects on the risk posed by organic contaminants such as PHE. Thus, we have shown that the common toxicity metric, EC₅₀ for springtail reproduction, was reduced by about 50% after five repeated heat bouts. Our results also show that the ability of springtail populations to persist during extreme heat waves is substantially lower if they are at the same time exposed to a toxicant like PHE. In the light of the climatic change we are facing, and the risk of more intense heat waves in the future, our study may give some of the answers to urgent questions, not just concerning risks from lipophilic compounds, but also a range of other contaminants.

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Figures

Figure 1. Survival of adult *Folsomia candida* exposed to ramping temperature from 20 °C to the target temperature at a rate of 4 °C hour⁻¹. Each point represents the mean $\pm$ SE ($N = 5$).

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671 Figure 2. Cumulative egg production (A) and time to first egg laying (B; percentage of 12 adults) of
 672 adult *Folsomia candida* during recovery after heat exposure. Prior to recovery at 20 °C, heat treated
 673 springtails were exposed to ramping temperature from 20 to 36 °C during 3 hours, and controls were
 674 kept at constant 20 °C. Each point represents the mean $\pm$ SE ($N = 12$).

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Figure 3. Survival (A) and fresh weight of adults (B) at the end of the 28-day combined heat and phenanthrene experiment (mean; $N = 6$). The numbers next to symbols indicate the concentration of phenanthrene in soil (mg kg^{-1} dry soil). To improve clarity, SE of the mean values are not shown in the graphs, but shown in Table S1B and Table S2B.

Figure 4. The number of juveniles microcosm⁻¹ at the end of the 28-day combined heat and phenanthrene experiment (mean; $N = 6$). The numbers next to symbols indicate the concentration of phenanthrene in soil (mg kg⁻¹ dry soil). To improve clarity, SE of the mean values are not shown in the graphs, but shown in Table S3B.

Figure 5. Effective Concentration of phenanthrene causing 50% reduction of juvenile production (EC_{50}) as a function of the number of heat cycles (A) and Effective Dose of Heat Cycles causing 50% reduction of juvenile production ($EDHC_{50}$) as a function of the concentration of phenanthrene in soil (B). Dashed lines indicate the 95% credibility intervals.

Figure 6. The concentration of phenanthrene in adult tissue at the end of the 28-day combined heat and phenanthrene experiment (mean; $N = 3$). To improve clarity, SE of the mean values are not shown in the graphs, but shown in Table S5.