

# Climate change and oil pollution: A dangerous cocktail for tropical zooplankton

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## ABSTRACT

Climate change and oil pollution pose a major threat to tropical marine ecosystems and to the coastal communities relying on their resources. The Gulf of Guinea is severely affected by multiple human induced stressors, but the potential impacts of these on marine productivity remain unknown. We investigated the combined effects of heatwaves (climate stressor) and the polycyclic aromatic hydrocarbon pyrene (proxy for oil) on the copepod *Centropages velificatus*. We quantified survival, reproduction and fecal pellet production of females exposed to concentrations of 0, 10, 100 and 100+ nM (saturated) pyrene under simulated heatwaves of different thermal intensity (+3 °C and +5 °C above control treatment temperature).

Thermal stress due to both moderate and intensive heatwaves resulted in reduced survival and egg production. The negative effects of pyrene were only measurable at the high pyrene concentrations. However, thermal stress increased the sensitivity of *C. velificatus* to pyrene, indicating a synergistic interaction between the two stressors.

We document that the interaction of multiple stressors can result in cumulative impacts that are stronger than expected based on single stressor studies. Further research is urgently needed to evaluate the combined impact of climatic and anthropogenic stressors on the productivity of coastal ecosystems, particularly in the tropical areas.

## 1. Introduction

The oceans are impacted by increasing temperatures and other anthropogenic stressors (Crain et al., 2008), and only 13 % of the world's ocean is considered to remain untouched by the impacts of humanity (Jones et al., 2018). Tropical oceans are particularly vulnerable, as many of the species in these areas live at the edge of their temperature tolerance (Nguyen et al., 2011), and many of the tropical areas suffer from multiple anthropogenic stressors (Halpern et al., 2015), resulting from various human activities. For instance, marine heatwaves are particularly severe in tropical regions (Intergovernmental Panel on Climate Change - IPCC et al., 2018), challenging the coastal productivity, biodiversity, ecosystems functions and services (Laffoley and Baxter, 2016; Cornwall, 2019). In addition to global warming, the marine environment is exposed to a wide range of other non-climatic stressors, such as overfishing, invasive species, chemical, oil or other potentially toxic elements, eutrophication and habitat loss (Chapman, 2016). For instance, oil pollutants, whether they derive from oil spills or

other chronic sources, have caused short- and long-term detrimental effects on marine zooplankton organisms (Buskey et al., 2016; Jiang et al., 2012) and generally, on marine ecosystems (Zhang et al., 2018; Walker et al., 2018).

The potential consequences of anthropogenic stressors on the marine environment are high for dense populations relying on coastal livelihoods, jeopardizing the health, food security and economic growth of these communities (Halpern et al., 2008; Allen, 2011; Intergovernmental Panel on Climate Change - IPCC et al., 2018). For instance, the Gulf of Guinea Large Marine Ecosystem (LME) is the most densely populated coastal zone in Africa (United Nations Statistics Division Demographic - UNSD, 2017), with over 81 million people directly depending on its vast resources, including fisheries, diverse habitats and energy resources (Global Environment Facility - GEF, 1998; Intergovernmental Oceanographic Commission of The United Nations Educational, Scientific and Cultural Organization - IOC-UNESCO and the United Nations Environmental Programme - UNEP, 2015). At the same time, the Gulf of Guinea LME is a hotspot of multiple stressors (Scheren

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and Ibe, 2002) and the states that confine it are among the regions with the lowest Ocean Health Index scores (Ocean Health Index - OHI, 2018). Besides climate change, the Gulf of Guinea LME is prone to multiple anthropogenic stressors, such as elevated levels of polycyclic aromatic hydrocarbons (PAHs) (Scheren and Ibe, 2002; Essumang, 2010), mainly deriving from oil exploitation and maritime pollution. The Gulf of Guinea is an important source of petroleum, producing approximately 2.7 million barrels per day (Energy Information Administration - EIA, 2019). Oil exploration activities have led to numerous oil spills in the Gulf of Guinea, the major ones being recorded in Nigeria and Cote d'Ivoire (International Tanker Owners Pollution Federation - ITOPF, 2018). 12,000 oil spill incidents were registered in Nigeria from 1976 to 2014, resulting in approximately 3 million barrels of oil spillage in the Niger Delta (Chinedu and Chukwuemeka, 2018). Oil spills in this area have shown to have a negative impact on macrozoobenthic communities (Zabbe and Uyi, 2014). The magnitude and cumulative effects of multiple stressors in the marine environment are poorly understood (Griffen et al., 2016). Laboratory studies manipulating multiple stressors in marine and coastal systems have both revealed strong synergistic interaction effects, and antagonistic or additive effects, sometimes even with the same stressor combinations (Crain et al., 2008). Also, the effects of climate change, a global persistent stressor (Intergovernmental Panel on Climate Change - IPCC et al., 2018), are rarely tested in combination with other anthropogenic stressors. Wenberg et al. (2011) reviewed marine climate change experiments and documented that the majority of these only tested single climatic stressors on targeted temperate organisms. While confirming the importance of addressing the combined effects of climate change and other stressors, Wenberg et al. (2011) pointed out the lack of studies on tropical and polar species and emphasized the need to include these in future experiments. Tropical and polar organisms are expected to be more sensitive to climate stressors, since they are acclimatized to extreme, but rather stable temperatures (Peck et al., 2004; Hoegh-Guldberg et al., 2007). Also, most of the current climatic driven studies are on benthic invertebrates (Wenberg et al., 2011), whereas studies on zooplankton, that play a key role in the marine food web and can serve as indicators of environmental stress (Parmar et al., 2016), are few.

The aim of this study was to investigate the combined effects of oil pollution and climate change on the survival and egg and pellet production of a common tropical copepod, by investigating the lethal and sublethal effects of pyrene (proxy for oil pollution) under different marine heatwave scenarios. The study was conducted in the coastal waters of Ghana, where the probability of marine heatwaves is predicted to increase (Frölicher et al., 2018) and the fast expanding crude oil production (Silverio González, 2016) adds to the already existing marine pollution. For instance, pyrene has both been measured in the coastal waters of Ghana and in commercially important fish species (Essumang, 2010; Nyarko et al., 2011; Bandowe et al., 2014). We hypothesized that 1) temperature and oil have synergistic detrimental effects on copepod survival and reproduction, 2) reproduction is more sensitive to synergistic effects than pellet production or survival and 3) the effects of stressors induce an abrupt change in ecophysiology and survival at a threshold concentration rather than a linear decrease in these variables. Understanding these interactive effects of marine heatwaves and oil pollution on the marine ecosystem is essential to establish research-based management, which is needed to promote marine conservation and prevent ecological and socio-economic impacts of environmental deterioration on the Gulf of Guinea.

## 2. Materials and methods

Experiments were conducted with the marine calanoid copepod *Centropages velificatus* (De Oliveira, 1947), an abundant species in the coastal waters of Ghana right after the major upwelling season (Bainbridge, 1960; West African Gas Pipeline - WAGP, 2004; Guinea Current Large Marine Ecosystem - GCLME, 2010). Female *C. velificatus* were

incubated under four different experimental conditions, over a 5-day period. The experimental conditions tested were: 1) a control without addition of stressors; 2) climate experiments with exposure to a heatwave; 3) oil pollution with exposure to pyrene; and 4) multi-stressor exposure to both heatwave and pyrene. Three different heatwave scenarios and four concentrations of pyrene were tested (Table 1). Survival was monitored daily by counting the numbers of dead copepods. Ingestion was estimated indirectly from fecal pellet production, and reproduction was investigated based on the production of eggs and nauplii. Nauplii were included in the measure of reproduction as hatching time in the high temperatures was shorter than the incubation time.

### 2.1. Copepods and algae

*C. velificatus* was collected at three occasions in October 2018, from the coastal waters of Cape Coast, Ghana (Table 2), 1.6–2.3 nautical miles off the fishing harbor of Elmina (5° 05'N, 1° 20'W). Copepods were sampled from the upper 5 m of the water column using a modified WP-2 plankton net (200 µm mesh with a 1-L non-filtering cod-end) while steaming at constant low speed (~1 knot). At the retrieval of the net, the content of the cod-end was immediately diluted into a 20-L thermo box filled with surface water. Surface seawater for the experiments was collected into 25-L plastic containers. After the return to the laboratory, no later than 1 h after sampling, both copepods and sea water were kept at the temperature of ca. 24 °C. In the laboratory, *C. velificatus* females were identified under a binocular microscope and individually transferred to an aerated container containing 40 µm filtered surface seawater from the sampling site. Copepods were fed *ad libitum*, at the concentration of ca. 400 µg C l<sup>-1</sup>, with the diatom *Thalassiosira weissflogii* from laboratory cultures. After isolation of the females an acclimatization period of 1 day was established to allow copepods to acclimate to laboratory conditions before undergoing the experimental work.

Cultures of *Thalassiosira weissflogii* were grown at 23 ± 1 °C, in 15-L plastic bags containing artificial seawater (salinity 32 ppt) enriched with B1 medium with vitamin (1.1 mL l<sup>-1</sup>), and silicate (1 mL l<sup>-1</sup>; Hansen, 1989). The cultures were maintained in exponential growth phase by diluting them daily. Cultures were constantly aerated and exposed to artificial light (2 pcs. Osram L, 36 W/840, Lumilux Cod white) positioned ~30 cm from the culture, in a photoperiod cycle of 16L: 8D. The diatom abundance in the culture was quantified daily by counting the number of cells in a Sedgewick Rafter counting chamber, using an inverted microscope (Olympus CK-2). The carbon content of *T. weissflogii* was assumed to be 131 pg C cell<sup>-1</sup> (Dutz et al., 2008). Additionally, the chlorophyll concentration of the cultures was measured daily on a fluorometer (Trilogy, Turner Designs, USA), and the cell counts were used to calibrate the fluorometer.

### 2.2. Experiments

#### 2.2.1. Treatments

For the climate stressor experiments, copepods were exposed to one of the three parallel experimental heatwave treatments: no heatwave (NO HW, 24.5 ± 0.5 °C), moderate heatwave (MHW, 27.5 ± 0.5 °C) and intense heatwave (IHW, 29.5 ± 0.5 °C). The sea surface temperature (SST) data recorded during the sampling period in the study site was used to define the NO HW treatment temperature. The temperature for MHW and IHW treatments was characterized following Hobday et al. (2018) marine heatwave categorization guidelines. MHW and IHW treatment temperatures corresponded to a 3 and 5 °C warmer SST, respectively, in relation to the 90th percentile of Ghana's SST recorded over a 30-year historical baseline period (1986–2015) during the major upwelling season (July to September). The frequency distribution of the recorded data (Ghana Meteorological Agency 2016) shows that most of the temperature data recorded during that period was distributed in the

**Table 1**

Overview of the experiments (NO HW= no heatwave, MHW= moderate heatwave, IHW= intense heatwave).

		Solvent	NO HW	MHW	IHW	No pyrene	10 nM pyrene	100 nM pyrene	100+ nM pyrene
1	Control	Water	•			•			
		Solvent	•			•			
2	Climate	MHW		•		•			
		IHW			•	•			
3	Oil pollution	10 nM pyrene	•	•			•		
		100 nM pyrene	•	•				•	
		100+ nM pyrene	•	•					•
		MHW, 10 nM pyrene	•	•			•		
		MHW, 100 nM pyrene	•	•				•	
4	Multistressor	MHW, 100+ nM pyrene	•	•					•
		IHW, 10 nM pyrene	•			•			
		IHW, 100 nM pyrene	•			•		•	
		IHW, 100+ nM pyrene	•			•			•

**Table 2**

Sampling station dates and coordinates.

Station	Date	Coordinates	Distance from harbour
ST1	03.10.2018	5° 03'N, 1° 19'W	1.6 M
ST2	20.10.2018	5° 02'N, 1° 20'W	2.1 M
ST3	26.10.2018	5° 03'N, 1° 20'W	2.3 M

thermal interval of 23.5–24.0 °C (Fig. S1). The 90th percentile was obtained by arranging the data in ascending order and using the following equation:

$$R = \frac{P}{100}(N + 1) \quad (1)$$

where  $P$  is the desired percentile and  $N$  is the number of points. The result,  $R$ , is the rank where the  $P$  is observed. The resultant 90th percentile for the 30-year historical baseline period (1986–2015) (Ghana Meteorological Agency 2016) is 24.6 °C, which closely agrees with the NO HW treatment temperature.

For the oil stressor experiments, individuals were exposed to pyrene solutions of 10, 100 and 100+ nM where the 100+ is a saturated pyrene concentration in seawater (Nørregaard et al., 2014). Pyrene exposure levels were selected to vary from a low expected impact to a lethal impact, based on previous studies on various physiological responses of copepods (Jensen et al., 2008; Hjorth and Nielsen, 2011; Nørregaard et al., 2014; Tøxværd et al., 2018). Pyrene (C<sub>16</sub>H<sub>10</sub>) was used as a proxy for oil since it is commonly found in the marine environments with high oil and shipping activities (Barata et al., 2005; Almeda et al., 2013; Tiselius and Magnusson, 2017). Pyrene has been shown to have a temperature dependent degradation over time (Grenvald et al., 2013) with higher reduction at higher temperatures. To ensure that the concentration of pyrene remained the same over the 5-day experiments, the exposure solutions were renewed daily.

Due to the high hydrophobic property of pyrene, stock solutions were made with acetone (≥99.8 %, Merck KGaA, Germany). Nominal stock solutions (0.1, 1 and 3 mM) were prepared weighing granulated pyrene (Sigma-Aldrich, purity N 99 %) on an analytical balance (Adventurer AX124, Ohaus, USA, readability 0.1 mg) and dissolving it into acetone (Table 3). Stock solutions were kept in 50 ml amber glass bottles in dark at 4 °C and each bottle was protected with aluminum foil to avoid phototoxicity, since the toxicity of PAHs such as pyrene increase

**Table 3**

Pyrene stock and exposure solutions used in the experiments.

Stock	Exposure solution	Content
1 (3 mM)	100+ nM	60.1 mg pyrene + 100 ml acetone
2 (1 mM)	100 nM	33.33 ml stock 1 + 66.67 ml acetone
3 (0.1 mM)	10 nM	10 ml stock 2 + 90 ml acetone
4 (0 mM)	0 nM	100 ml acetone (control experiment)

by exposure to natural or artificial sunlight via photosensitization and/or photomodification reactions (Bellas and Thor, 2007; Björn and Huovinen, 2008). An additional solvent control was included in experiments to test whether acetone had any effect on the survival or egg production of copepods.

### 2.2.2. Set up

Experiments were conducted between the 5th of October and 1st of November 2018. Each experiment lasted 5 days and included a control. Each treatment contained 4 replicates. As an exception and due to contamination originated from the water containers, the experiments with pyrene as a single stressor were run for 3 days, using bottles of 0.33 L and 5 individuals per bottle.

The climate scenarios were established in the laboratory in three 80-L water pools, each corresponding to one temperature (heatwave) treatment. The desired temperatures were established using a thermostatic heater (EHEIM thermocontrol 200, Germany, ± 0,5 °C accuracy) installed in each of the pools. The pools were constantly aerated using an air pump to ensure that the water temperature was even in the pool. The temperature in the pools was monitored every 15 min using temperature loggers (UTBI-001, Onset, USA, accuracy ± 0.21 °C) (Fig. 1).

Fresh food suspensions were prepared daily. At the beginning of each run a stock solution was prepared from aerated filtered seawater media and 400 µg C L<sup>-1</sup> of the diatom *Thalassiosira weissflogii*. Twenty 1.2-L acid washed glass bottles were filled with the stock solution, and kept at the experimental temperatures for 2 h before adding the copepods. For the oil experiments, 118 µl of the previously prepared stock solutions were added into the bottles to make the exposure solutions of 10, 100 or 100+ nM pyrene. Similarly, 118 µl of acetone were added to the solvent control bottles. Bottles were rotated several times to ensure that the pyrene and solvent were well distributed, before gently adding 7 female *C. velificatus* into each of the bottles. The bottles were placed into the water baths, and manually rotated 5 times a day. The experiments were conducted under a photoperiod cycle of 16L: 8D.

Every day, copepods were transferred to clean bottles containing fresh food suspension and solvent or pyrene solutions. The copepods were gently filtered onto a submerged 200 µm mesh sieve and carefully rinsed into a Petri dish containing filtered seawater. The number of dead individuals were noted and removed, and the live individuals were transferred to the new bottles and brought back to the thermal treatment pools. An individual was considered dead when it was partially or totally degraded and/or had no reaction to physical stimuli. After transfer of the copepods, contents of the bottles were sieved onto a 20 µm mesh size nets, and flushed into Petri-dishes. Samples were fixed in 2 % acidified Lugol, and the fecal pellets, eggs and nauplii were counted under a stereo microscope (Olympus SZ61, Japan).

At the end of the experiments, copepods, fecal pellets and eggs from each incubation bottle were preserved in 2 % acidified Lugol solution in 20 ml glass vials for size measurements. Copepod prosome lengths were measured using a calibrated ocular micrometer with a precision of 20

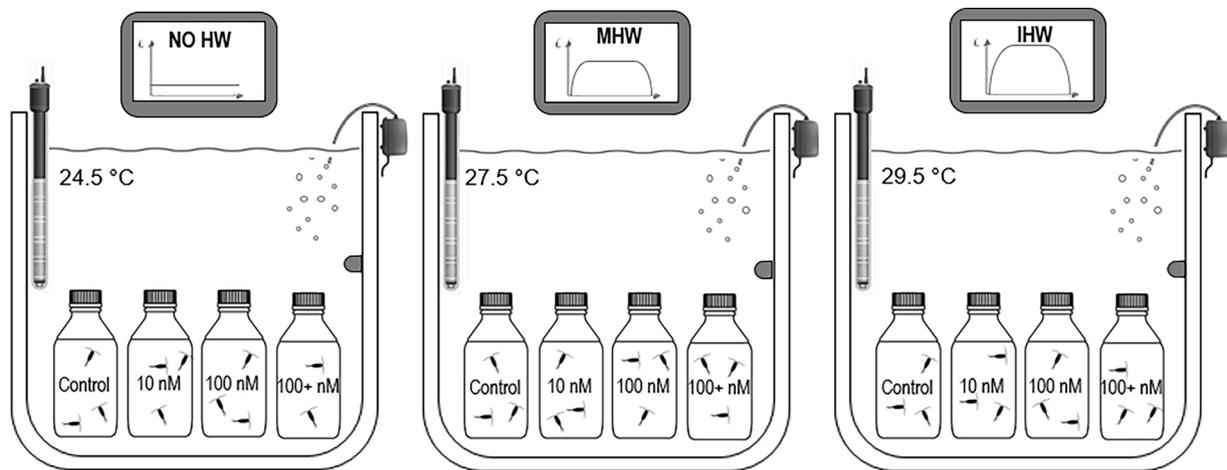


Fig. 1. Schematic of experimental setup consisting in three water pools, containing each the treatment bottles (a), a thermostatic heater (b), an air pump (c), and a temperature logger (d).

µm, under an inverted microscope (Olympus CK2, optic LWD C, A4PL). Eggs were measured for diameter and pellets for length and width using an inverted microscope with a precision of 5 µm (Table 3). Treatment bottles, filters and other tools exposed to pyrene contaminated water were rinsed after use with a sequence of acetone, fresh water, and filtered seawater.

### 2.3. Data analysis

Female survival was estimated using the Kaplan-Meier method (Kaplan and Meier, 1958), to determine whether an experimental marine heatwave and/or pyrene exposure influences survival:

$$S(t) = \prod_i \left(1 - \frac{d_i}{n_i}\right) \quad (2)$$

where  $S$  is the probability of survival at the given time  $t$ ,  $d_t$  is the number of females who die during time period  $t$ , and  $n_t$  is the number of females at risk at the beginning of time period  $t$ . This method accounts also for censored observations. Survival curves were compared using the log-rank test. Survival tests were performed using GraphPad Prism 8.0.2.

The daily fecal pellet production rates (PP: Pellets individual<sup>-1</sup> d<sup>-1</sup>) and the combined egg and naupliar production rates (EP: egg + nauplii individual<sup>-1</sup> d<sup>-1</sup>) were estimated for each incubation bottle. PP and EP were converted to weight-specific rates (µg C µgC<sup>-1</sup>) based on the measured female prosome lengths and the calculated volumes of eggs and fecal pellets (Table 4). Egg carbon was estimated assuming a sphere and a volume to carbon ratio of 0.14 pg C µm<sup>-3</sup> (Kjørboe et al., 1985), and female carbon biomass was calculated from the length to carbon

regression of Satapoomin (1999). Pellet carbon was calculated from the pellet volume, assuming a cylinder and a volume to carbon ratio of  $4.3 \times 10^{-8} \mu\text{g C } \mu\text{m}^3$  (Swailethorp et al., 2011). A 16.5 % shrinkage of the copepods, eggs and fecal pellets due to the use of acidified Lugol solution was assumed (Jasper and Carstensen, 2009). Since there were no significant differences on egg and pellet sizes between the different experiments (data not shown), measurements from all experiments were pooled, with an exception of one experiment, where pellet sizes were slightly smaller due to smaller collected individuals.

Daily rates of PP and EP were used for the statistical analysis to avoid day interdependency from cumulative values. Linear mixed effects analysis of the relationship between oil and climate stressor treatments, exposure days and experiment runs were performed in R 3.4.3 (R Core Team, 2012) using lme4 package (Bates et al., 2012). Models were compared in a likelihood ratio test using analysis of variance (ANOVA). The initial linear mixed effect model for PP and EP was outlined as:

$$y_i = \alpha(\text{pyr}_i, d_i, H_i) + \delta(\text{Sol}_i) + Z(R_i) + \epsilon_i \quad (3)$$

where  $y_i$  is the observed response of PP or EP;  $\alpha$  is the interaction of pyrene,  $\text{pyr}$ , day,  $d$ , and heatwave,  $H$ , effect;  $\delta$  is the effect of solvent,  $\text{sol}$ ;  $Z$  is the random effect of runs,  $R$ , and  $\epsilon$  is the normal noise error term.  $R$  (the different experimental setup dates) was considered to have an effect on PP and EP due to the different starting times of the experimental runs, which involve copepod specimens from different collection dates, and possible differences in seawater quality. Similarly, the inter-dependence between  $H$  and  $d$ , and  $\text{pyr}$  and  $d$  was tested by step-wise comparison and reduced from the model if no significant interaction was found. The final reduced models for PP and EP are presented in Results.

Table 4

Female prosome lengths (µm), average egg diameter (µm) and egg and pellet volume (µm<sup>3</sup>; mean ± SD).  $n$  indicates number of measured samples. (\*) indicates pellet measurements from experiment with smaller individuals (see Methods).

Pyrene treatment	Prosome length (µm) Mean ± SD	$n$	Female carbon content (µg)
0 nM	1385 ± 84	30	21.1
10 nM	1363 ± 75	31	19.9
100 nM	1364 ± 77	14	20.0
100 nM	1054 ± 111	15	7.5
100+ nM	1092 ± 105	20	8.5
<b>Egg diameter Mean ± SD</b>	<b>Egg volume (µm<sup>3</sup>) Mean ± SD</b>	<b><math>n</math></b>	<b>Egg carbon content (µg)</b>
78.3 ± 11.4	$1.96 \times 10^5 \pm 8.16 \times 10^4$	69	0.03
	<b>Pellet volume (µm<sup>3</sup>) Mean ± SD</b>	<b><math>n</math></b>	<b>Pellet carbon content (µg)</b>
	$4.62 \times 10^5 \pm 2.65 \times 10^4$	125	0.019
	$3.74 \times 10^5 \pm 1.76 \times 10^4$ (*)	125	0.016

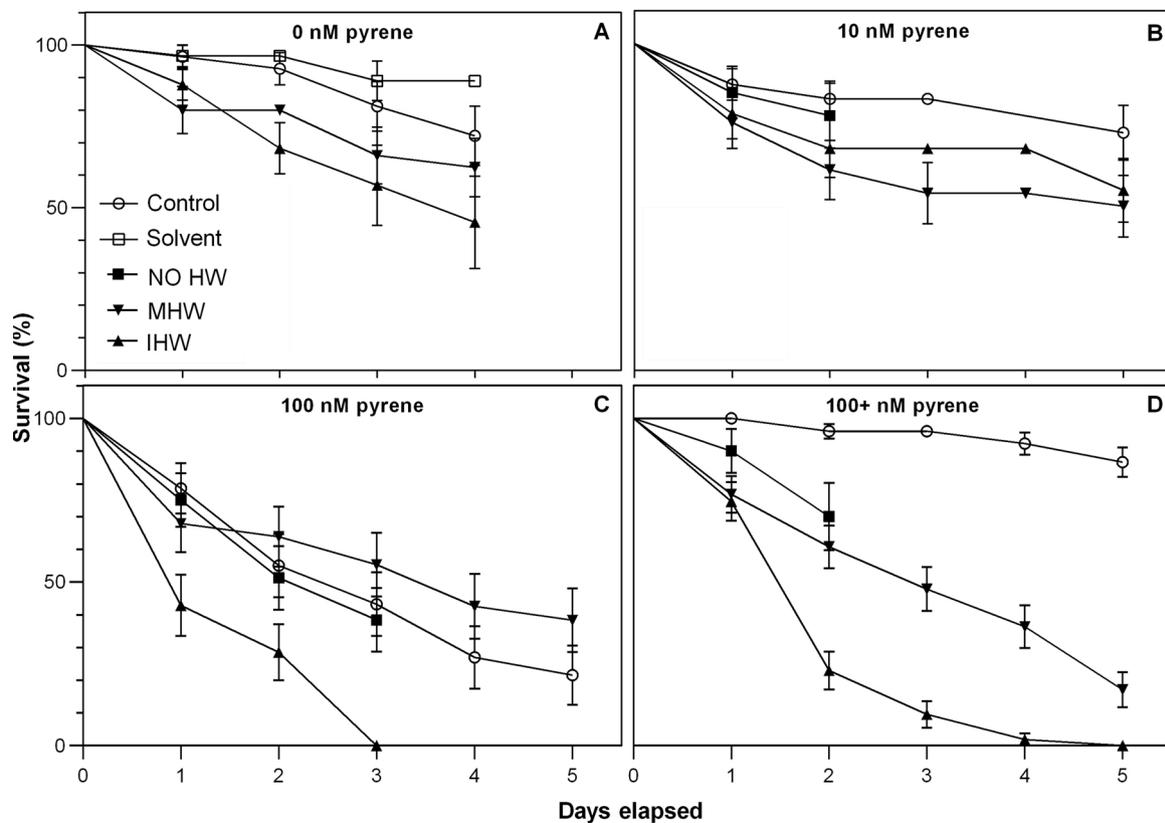


Fig. 2. Daily survival of *Centrophages velificatus* females in untreated seawater (control), solvent control and exposures to 0 (A), 10 (B), 100 (C) and 100+ (D) nM pyrene, under the three thermal scenarios. (NO HW,  $24.5 \pm 0.5$  °C) no heatwave, (MHW,  $27.5 \pm 0.5$  °C) moderate heatwave, (IHW,  $29.5 \pm 0.5$  °C) intense heatwave (%; mean  $\pm$  SE). Note that the experiments with pyrene as a single stressor were only run for 3 days (see Methods).

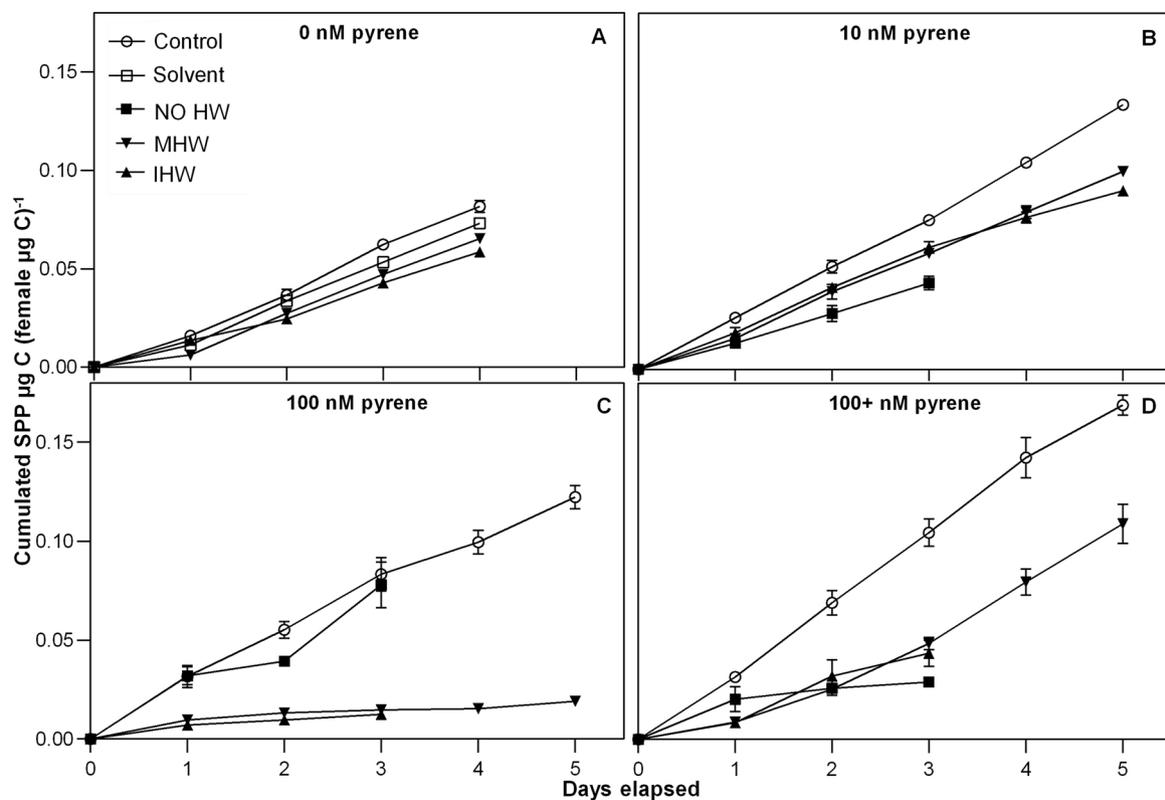


Fig. 3. Cumulative specific fecal pellet production SPP;  $\mu\text{g C } \mu\text{g C}^{-1}$  of *C. velificatus* females in control, solvent control, and exposed the different heatwaves and pyrene concentrations (mean  $\pm$  SE). Abbreviations as in Fig. 2.

### 3. Results

#### 3.1. Survival

Survival of the copepods declined under heatwave exposure ( $p < 0.01$ , Fig. 2A). However, there were no statistically significant differences between the MHW and IHW in the absence of pyrene ( $p > 0.05$ ), although the decline in survival was more accentuated in the intense heatwave treatment (IHW;  $p < 0.001$ ; MHW;  $p < 0.05$ ).

In the absence of the climate stressor, survival was not significantly different under the exposure to 10 or 100 nM pyrene ( $p > 0.05$ ; Fig. 2B), but a significant decline was observed in the +100 nM treatment ( $p < 0.0001$ , Fig. 2D). Heatwaves did not significantly reduce survival in the 10 nM pyrene treatment ( $p > 0.05$ ). However, the lowest copepod survival was observed in the 100 nM ( $p < 0.0001$ ; Fig. 3C) and 100+ nM pyrene exposure experiments ( $p < 0.0001$ ; Fig. 2D) in combination with the IHW. On the last exposure day with 100+ nM pyrene, there were no survivors in the IHW treatment, and only 17 % survived the MHW treatment. The survival in the 100 nM exposure, was significantly lower only in combination with IHW ( $p < 0.001$ ). While the mortality in the single stressor experiments was more or less linear, the combination of high concentrations of pyrene and IHW induced highest mortality on the first few days of the experiments (Fig. 2). There were no statistically significant differences in the survival between solvent and water controls ( $p > 0.05$ ).

#### 3.2. Pellet production

The linear mixed effect model for PP was reduced to two fixed effects and a random effect for model simplification (Table 5). The final model was accordingly reduced as follows:

$$y_i = \alpha(\text{pyr}_i, H_i) + \beta(\text{pyr}_i, d_i) + Z(R_i) + \varepsilon_i \quad (4)$$

where  $y_i$  is the vector-value random response of PP,  $\alpha$  is the interaction of pyrene, pyr, and heatwave,  $H_i$ ;  $\beta$  is the interaction of pyrene and days,  $d_i$ ;  $Z$  is the random effect of the experiment runs,  $R_i$ ; and  $\varepsilon$  is the normal noise error term. Specific PP was not affected by a heatwave exposure in the absence of pyrene ( $p > 0.05$ , Fig. 3A) or at the exposure to 10 nM pyrene ( $p > 0.05$ , Fig. 3B). However, although the exposure to 100 nM pyrene did not affect the specific PP as a single stressor, there was a significant response in PP when the copepods were exposed to a combination of 100 nM pyrene and a heatwave ( $p < 0.0001$ ), showing a strong reduction of PP in both the MHW and IHW treatments (Fig. 3C). In contrast, the exposure to 100+ nM pyrene had a significant negative effect on specific PP as a single stressor ( $p < 0.0001$ ), but the effect was not intensified by the exposure to a heatwave (Fig. 3D). The synergistic effect of oil and climate stressor at 100 nM pyrene exposure was evident in the average PP (Fig. 4), which was reduced by 86 % in the MHW and IHW experiments ( $4 \pm 1$  pellets female<sup>-1</sup> d<sup>-1</sup>) compared to the control ( $30 \pm 6$  pellets female<sup>-1</sup> d<sup>-1</sup>). While there was not a

**Table 5**

Model reduction process for pellet production linear mixed effect analysis. The final reduced model is emphasized in bold.

Fit	Model	Interactions	Result
a	$y_i = \alpha(\text{pyr}_i, d_i, H_i) + \delta(\text{Solv}_i) + Z(R_i) + \varepsilon_i$	Initial model	
b	$y_i = \alpha(\text{pyr}_i, H_i) + \beta(\text{pyr}_i, d_i) + \gamma(H_i, d_i) + \delta(\text{Solv}_i) + Z(R_i) + \varepsilon_i$	Third-order	Accepted ( $p > 0.05$ )
c	$y_i = \alpha(\text{pyr}_i, H_i) + \beta(\text{pyr}_i, d_i) + \gamma(H_i, d_i) + Z(R_i) + \varepsilon_i$	Solvent, solv	Accepted ( $p > 0.05$ )
d	$y_i = \alpha(\text{pyr}_i, H_i) + \beta(\text{pyr}_i, d_i) + Z(R_i) + \varepsilon_i$	Heatwave, H, days, d	Accepted ( $p > 0.05$ )
e	$y_i = \alpha(\text{pyr}_i, H_i) + Z(R_i) + \varepsilon_i$	Pyrene, pyr, days, d	Rejected ( $p < 0.001$ )

significant inter-dependence effect between the heatwaves and time (experimental days;  $p > 0.05$ ), the effect of pyrene was significantly increased by the incubation time ( $p = 0.001$ ; Fig. 3). The effect of solvent on PP was not statistically significant ( $p > 0.05$ ) from the control.

#### 3.3. Egg production

The linear mixed effect model for EP was reduced to three fixed effects and a random effect for model simplification (Table 6). The final model was then reduced as follows:

$$y_i = \alpha(\text{pyr}_i, H_i) + \beta(\text{pyr}_i, d_i) + \delta(\text{Solv}_i) + R(R_i) + \varepsilon_i \quad (5)$$

where  $y_i$  is the vector-value random response of EP and  $\delta$  is the interaction of solvent, solv.

Specific EP declined under heatwave exposure ( $p > 0.029$ ; Fig. 5A). MHW and IHW treatments without pyrene resulted in a reduction of average EP by 37 % ( $6 \pm 2$  eggs female<sup>-1</sup> d<sup>-1</sup>) and 32 % ( $7 \pm 2$  eggs female<sup>-1</sup> d<sup>-1</sup>) respectively, in relation to the control ( $11 \pm 2$  eggs female<sup>-1</sup> d<sup>-1</sup>; Fig. 6). EP was not affected by the exposure to 10 nM pyrene, but the exposure to 100 nM pyrene and 100+ nM significantly reduced the specific EP ( $p < 0.01$ , Fig. 5C and D). However, in contrast to the pellet production and survival, heatwaves did not significantly influence the response of the egg production to pyrene, although the average EP was 73 % lower in the 10 nM pyrene treatment combined with IHW ( $2 \pm 1$  eggs female<sup>-1</sup> d<sup>-1</sup>) compared to the control ( $10 \pm 2$  eggs female<sup>-1</sup> d<sup>-1</sup>). Overall, the effect of high concentrations of pyrene on EP was, however, not significantly altered by the incorporation of a climate stressor. Similar to pellet production, the effect of the heatwave treatment did not change between the days ( $p > 0.05$ ), but the effect of pyrene was significantly affected by the incubation time ( $p = 0.001$ ). In contrast to PP, the solvent influenced EP ( $p < 0.01$ ), and the reduction of EP due to pyrene was in most cases similar to the reduction due to the solvent (Figs. 5 and 6).

## 4. Discussion

We investigated whether thermal and pyrene stress have negative impacts on the survival, fecal pellet production and reproductive success of a tropical copepod species. While heatwaves as a single stressor reduced the survival and egg production of copepods, pyrene in the concentrations  $\geq 100$  nM generally had a detrimental effect on survival, pellet production and egg production. Also, the detrimental effects of pyrene on the survival and egg production were intensified by the exposure to a heatwave, to a degree that was higher than what would be expected based on the single stressor experiments. Our results thus indicated synergistic effects of an exposure to a high concentration of pyrene and a heatwave.

#### 4.1. Tolerance to temperature and pyrene as single stressors

The observed lethal and sublethal effects of temperature on *C. velificatus* agree with other experimental studies that have observed declines in survival and reproduction in harpacticoid copepods exposed to thermal stress (Koch et al., 2017; Siegle et al., 2018). Splashpool harpacticoids can tolerate a wide range of temperature fluctuations during the day (Burton et al., 1979), yet, an increase of temperature prolonged over time results in lethal and sublethal effects (Siegle et al., 2018). Negative effects of a 4 °C increase of temperature on the reproductive success of tropical copepods have also been documented by Doan et al. (2019), and similarly, Rhyne et al. (2009) showed that an increase of 2–4 °C resulted in declines in survival and reproduction. A 6 °C increase of temperature from a coastal power plant showed significantly reduced the grazing rates of tropical copepods (Hu et al., 2018). In comparison, our experiments showed no negative effects on pellet production, but similar declines in reproduction and survival at 3–5 °C

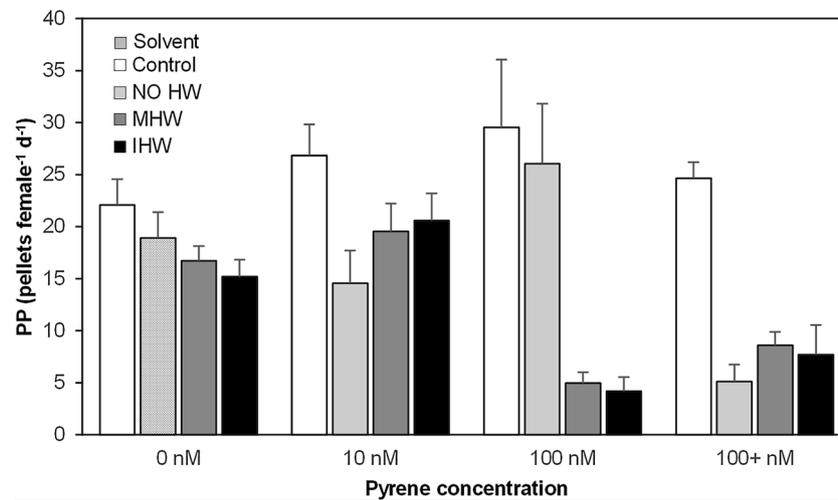


Fig. 4. Average fecal pellet production (PP, pellets female<sup>-1</sup> day<sup>-1</sup>) of *C. velificatus* females in control, solvent control and exposure to the heatwaves and pyrene concentrations, during the first three days of experiment (mean  $\pm$  SE). Abbreviations as in Fig. 3.

Table 6

Model reduction process for egg production linear mixed effect analysis. The final reduced model is emphasized in bold.

Fit	Model	Interactions	Result
a	$y_i = \alpha(\text{pyr}_i, d_i H_i) + \delta(\text{Solv}_i) + Z(R_i) + \epsilon_i$	Initial model	
b	$y_i = \alpha(\text{pyr}_i, H_i) + \beta(\text{pyr}_i, d_i) + \gamma(H_i, d_i) + \delta(\text{Solv}_i) + Z(R_i) + \epsilon_i$	Third-order	Accepted ( $p > 0.05$ )
c	$y_i = \alpha(\text{pyr}_i, H_i) + \beta(\text{pyr}_i, d_i) + \gamma(H_i, d_i) + Z(R_i) + \epsilon_i$	Solvent, <i>solv</i>	Rejected ( $p < 0.001$ )
d	$y_i = \alpha(\text{pyr}_i, H_i) + \beta(\text{pyr}_i, d_i) + \delta(\text{Solv}_i) + Z(R_i) + \epsilon_i$	Heatwave, <i>H</i> , days, <i>d</i>	Accepted ( $p > 0.05$ )
e	$y_i = \alpha(\text{pyr}_i, H_i) + \delta(\text{Solv}_i) + Z(R_i) + \epsilon_i$	Pyrene, <i>pyr</i> , days, <i>d</i>	Rejected ( $p < 0.001$ )

increase of temperature.

Marine heatwaves have the potential to alter species composition, diversity, abundance and productivity and it is predicted that these events will hamper native species in favour of non-native species that can tolerate a wider thermal window (Sorte et al., 2010). This can have serious implications for native tropical species. Recent studies have shown that the impact of marine heatwaves is more severe for species living near their thermal limit (Smale et al., 2019) and tropical marine organisms have shown to be less tolerant to marine heatwaves and to have lower acclimation response than temperate organisms (Vinagre et al., 2016, 2018). This confirms that tropical species, although capable to withstand higher temperatures, have lower thermal plasticity and live closer to their thermal limits (Somero, 2010; Nguyen et al., 2011), making them more vulnerable to climate change (Pörtner and Farrell, 2008; Tewksbury et al., 2008).

Our results confirm the vulnerability of calanoid copepods to pyrene exposure (Nørregaard et al., 2014; Krause et al., 2017; Tøxværd et al., 2018), although lethal effects were only observed at the saturating concentration. The mechanisms of pyrene toxicity involve its interaction and interference with membrane and cellular processes, causing long-term damage to cellular structures (Neff, 1979). The effects of pyrene toxicity in copepods include mortality (Bellas and Thor, 2007), narcosis (Almeda et al., 2013) and reduced ingestion and reproduction rates (Jensen et al., 2008). Narcotic effects of pyrene on copepods at concentrations 30 % lower than the estimated LC<sub>50</sub> have also been observed by Barata et al. (2005). We did observe, but not quantify, reduced motility at high pyrene concentrations, which could indicate a narcotic effect of pyrene. A prolonged narcosis would eventually lead to

mortality.

In our experiments, survival of *C. velificatus* was not affected at pyrene concentrations of 10 and 100 nM if the temperature was not increased. This agrees with a recent study from Krause et al. (2017), where *Acartia tonsa*, a common and widely distributed neritic copepod, was exposed to 10 nM pyrene over two generations without showing significant lethal effects, whereas a high mortality was observed at saturated pyrene concentrations. The reported lethal effects by Krause et al. (2017) at 100 nM concentrations were only significant in the first generation of *A. tonsa*, indicating that the tolerance to pyrene exposure increased between the generations. We did not observe significant lethal effects at 100 nM pyrene concentrations, and it could be argued that the *C. velificatus* had developed a tolerance to pyrene due to its presence in the coastal waters of Ghana (Essumang, 2010; Nyarko et al., 2011; Bandowe et al., 2014).

The physiological responses of tropical marine organisms differ from temperate and Arctic species (Ursin, 1984). For instance, aspects such as size, growth rate, feeding strategy, lipid content or the rates and mechanisms of elimination and uptake of PAHs (passively, controlled by diffusion; and actively, through ingestion) will regulate their toxicity (Meador et al., 1995). As an example, different tolerances to oil exposure have been observed among different Arctic calanoid species (Hjorth and Nielsen, 2011) and different gelatinous zooplankton species (Almeda et al., 2013). The influence of lipids on the toxic effects of lipophilic compounds in aquatic organisms has been discussed by several authors (Lassiter and Hallam, 1990; Lotufo, 1998; Hjorth and Nielsen, 2011). The lipophilic nature of PAHs makes them bind to and accumulate in storage lipids, postponing their toxic effect (Walker, 2009). Consequently, tropical species could be less tolerant to pyrene than arctic species, since they will on average contain less lipids (Kattner and Hagen, 2009). The effects of oil on marine zooplankton will also depend on the nature, type and amount of oil, and on environmental conditions such as temperature, light, salinity or winds (Meador et al., 1995).

#### 4.2. Effects of multiple stressors

Our results agree with the previous multiple stressor experiments subjecting Arctic calanoid copepods to 100 nM pyrene concentrations at different temperature. These studies showed that an increase in temperature exacerbates the negative effects of pyrene on naupliar survival (Grenvald et al., 2013) and ingestion and reproduction of females (Hjorth and Nielsen, 2011). Generally, studies on the multiple stressor effects of oil and climate change are scarce and have up to date primarily focused on Arctic organisms. Our results provide the first account of the

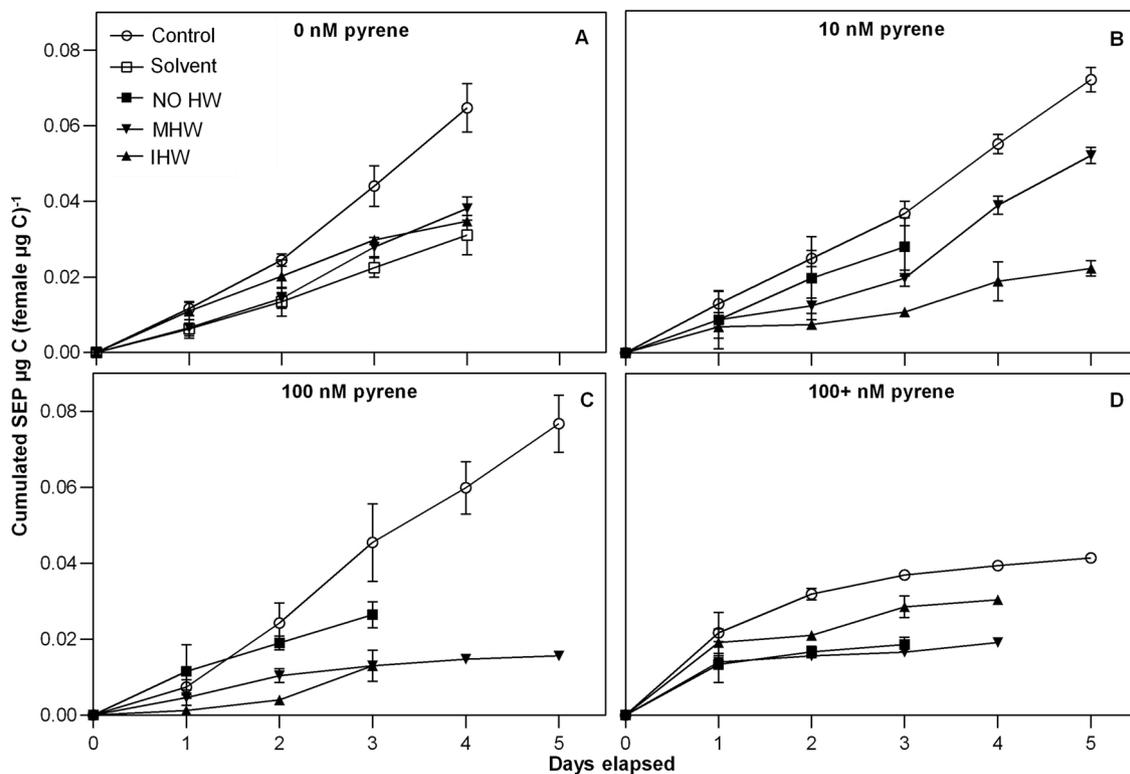


Fig. 5. Cumulative specific egg production SEP;  $\mu\text{g C } \mu\text{g C}^{-1}$  of *C. velificatus* females in control, solvent control, and exposed to the different heatwaves and pyrene concentrations (mean  $\pm$  SE). Abbreviations as in Fig. 3.

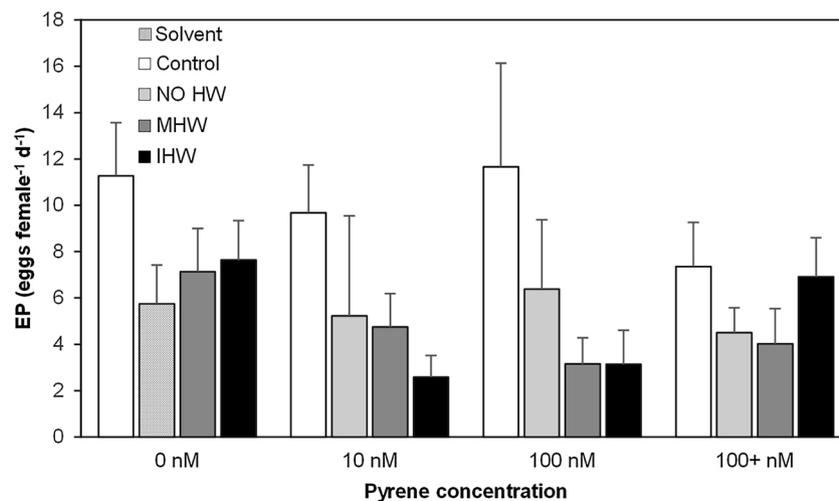


Fig. 6. Egg production (EP, eggs female<sup>-1</sup> day<sup>-1</sup>) of *C. velificatus* females in control, solvent control and exposure to the heatwaves and pyrene concentrations, during the first three days of experiment (mean  $\pm$  SE). Abbreviations as in Fig. 3.

combined effects of oil and temperature on tropical copepods, suggesting that the overall synergistic interactions are similar to Arctic copepods.

Studies testing multiple stressors have shown a highly variable interaction among the stressors, underlining the challenge of these type of studies (Crain et al., 2008). Estimating the effects of multiple stressors is difficult due to the different mechanisms of action on the organisms (Sokolova, 2013) and the complexity of incorporating different order of the interactions of the stressors (Billick and Case, 1994). Jiang et al. (2012) observed that the tolerance of subtropical copepods to crude oil decreased during the warmer seasons, indicating a temporal pattern in tolerance to temperature stressor. This demonstrates that changes in

temperature, even within the normal seasonal variation, may affect the organism tolerance to pollutants (Neff, 1979), highlighting that studies should no longer focus on single stressors only.

Here, we worked with a cocktail of stressors: oil pollution and climate change and, while their impact on tropical regions remain unknown, studies in the Arctic suggest that the combined effects of oil pollution and increased temperatures will alter the relative copepod abundance (Dinh and Nielsen, 2016). Similarly, a recent multi-model study showed that the combination of PAHs and especially temperature, are important predictors of copepod abundance and diversity in the Belgian part of the North Sea (Deschutter et al., 2017). Even though the model of Deschutter et al. (2017) was applied for temperate calanoid

species, it could be considered a conservative estimate for tropical zooplankton with more restrictive temperature regulating roles. Our study suggests that due to the observed reduction in the reproductive output of *C. velificatus* the effects of heatwaves in combination with pyrene could also have implications on tropical copepod abundance and potentially, the secondary production in the Gulf of Guinea LME.

#### 4.3. Energetic trade-offs

The observed declines in survival, ingestion and reproductive rate of *C. velificatus* under the multiple stressor exposure could be attributed to energetic trade-offs. Under no stress, the energy obtained from ingestion is optimally allocated between growth, reproduction and survival (Lichtman et al., 2013). Under stress, the aerobic scope, which represents the surplus energy that is left after basal maintenance costs (Guderley and Pörtner, 2010), is reduced and the optimal energy budget is rearranged to ensure the main priority: survival. A moderate stress will reduce the aerobic scope, allocating more energy to protect and repair the damage caused by the stressor (Sokolova, 2013). This could explain the declines in egg production observed in the heatwave stressor experiment (Fig. 6A), which cannot be associated to a decline in ingestion rates, since there was no decline in pellet production, but could be a result from a lower energy investment in reproduction. The same pattern was observed in the experiments with 100 nM pyrene exposure, where the decrease in egg production was not accompanied by a decrease in pellet production (Figs. 4C, 6 C), unless the pyrene exposure was combined with a heatwave treatment. As the stressor intensifies, more energy and metabolic capacity will be dedicated to ensure survival (Sokolova, 2013). Under thermal stress, the organism may undergo metabolic depression to increase the chances of survival until the situation improves (Pörtner and Farrell, 2008), at a cost of reproduction, growth and activity. The thermal stress at high pyrene concentrations could have activated this mechanism, explaining the significant declines observed in pellet production (Fig. 3C) and egg production (Fig. 5C). The low survival rates observed in these treatments (Fig. 2C and D) could be explained by the acute toxic effect of pyrene, but also by a negative aerobic scope that can no longer cover basal demands (Sokolova, 2013). While our results seem to agree with energetic trade-offs, experimental demonstration of energetic trade-offs was out of the scope of our work. Nevertheless, the concept of energy-limited tolerance to stress can be useful for evaluating the effects of multiple stressors in zooplankton, as proposed by Sokolova (2013), using energy balances to define the effects of multiple stressors and distinguish between moderate and intense stress.

#### 5. Conclusions

Investigating the impact of multiple stressors in the Gulf of Guinea is important given the ecological and socio-economic importance of a healthy marine ecosystem in the area (McGlade et al., 2002). Marine and coastal resources are a significant source of economic activity for Ghana: a major artisanal fishing nation (Atta-Mills et al., 2004), that strongly relies on fish as a protein source (Food and Agriculture Organization - FAO, 2016). However, Ghanaian fisheries are overexploited as a result of increasing fishing pressure, poor law enforcement and illegal fishing practices (Afoakwah et al., 2018). For instance, the use of petrol or diesel is reported to be a fishing practice, used either by pouring it on the sea (according to fishermen the water becomes more transparent, helping them to spot fish) or by mixing it with a powdered detergent which attracts fish and causes their incapacitation after ingestion, making them easier the capture (Afoakwah et al., 2018). These local pollution sources add to the effects of oil exploration, industry, shipping, house-hold waste and climate change.

We demonstrated vulnerability of tropical zooplankton to oil pollution and marine heatwaves, emphasizing that the negative effects of heatwave and pyrene exposure on tropical zooplankton aggravate when

the two stressors interact. We suggest that the effects of heatwaves in combination with oil (pyrene) could have implications on tropical copepod abundance, secondary production and potentially the fish stock with large implication for the livelihood of people dependent marine resources from the Gulf of Guinea LME. As the tropical coastal nations in Africa and Asia are expected to be the countries to suffer the most severe effects from climate change, the effects of multiple stressors at the basis of the food webs are not trivial, but can have major implications for sustainable development, food security, livelihoods and economic growth (Intergovernmental Panel on Climate Change - IPCC et al., 2018) in these countries, where the rapid population growth is further increasing their dependency on coastal and marine resources (Allen, 2011).

#### CRedit authorship contribution statement

**Laura Hernández Ruiz:** Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft, Writing - review & editing. **Bernard Ekumah:** Investigation, Methodology, Writing - review & editing. **Delove Abraham Asiedu:** Investigation, Methodology, Writing - review & editing. **Giovanna Albani:** Investigation, Methodology, Writing - review & editing. **Emmanuel Acheampong:** Investigation, Methodology, Writing - review & editing. **Sigrún H. Jónasdóttir:** Investigation, Methodology, Writing - review & editing. **Marja Koski:** Investigation, Methodology, Writing - review & editing. **Torkel Gissel Nielsen:** Conceptualization, Writing - review & editing, Project administration, Funding acquisition.

#### Declaration of Competing Interest

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

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#### Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.aquatox.2020.105718>.

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