Consequences of combined exposure to thermal stress and the plasticiser DEHP in *Mytilus* spp. differ by sex

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Abstract

Little is known about the combined effect of environmental factors and contaminants on commercially important marine species, and whether this effect differs by sex. In this study, blue mussels were exposed for seven days to both single and combined stressors (i.e., +3°C elevated temperature and two environmentally relevant concentrations of the plastic softener DEHP) in a factorial design. Males were observed to be more sensitive to increased temperature, demonstrated by the significant increase in out-of-season spawning gonads and increased gene expression of the antioxidant catalase and the estrogen receptor genes. On the other hand, while the gametogenesis cycle in females was more resilient than in males, DEHP exposure altered the estrogen-related receptor gene expression.

We show that the combined stressors DEHP and increased temperature, in environmentally relevant magnitudes, have different consequences in male and female mussels, with the potential to impact the timing and breeding season success in *Mytilus* spp..

Keywords

DEHP, global warming, gametogenesis cycle, stress response, estrogen receptor-like, multiple stressors

1. Introduction

Mytilus spp. are commonly utilised as sentinel organisms in biomonitoring programmes worldwide due to their cosmopolitan distribution along coastal waters and their high tolerance to several toxic compounds (Gorbi et al., 2008; Markert et al., 2003; Viarengo et al., 2007). Additionally, considering that the global harvest of the blue mussel is reported to have reached more than 200,000 tonnes in 2018 (FAO 2018), mussels are used as bioindicators for health and food safety (Chiesa et al., 2018; Van Cauwenberghe and Janssen 2014).

The reproductive cycle of British mussels has been extensively examined in the past decades (Chapman et al., 2017; Lowe et al., 1982; Secor et al., 2001; Seed 1969). The dependency of mollusc gametogenesis on geographic distribution (Virgin and Barbeau, 2017), environmental conditions (Fearman and Moltschaniwskyj 2010), nutrient availability (Kang et al., 2006), and endocrine disruptor presence (Siah et al., 2003) is likewise well known. Furthermore, the reproductive cycle of molluscs is dissimilar between males and females, in terms of timing for reaching ripe and resting stages (Mladineo et al., 2007; Sunila et al., 1981), number of gametes released at different time points of the spawning season (Anantharaman et al., 2012), and estrogen and androgen content (Smolarz et al., 2018).

Plastic, an affordable and durable material, is a primary commodity for the manufacture of everyday products (Andrady 2011; Thompson et al., 2009). The resulting environmental contamination of terrestrial (de Souza Machado et al., 2018; Ragusa et al., 2021; Schwabl et al., 2019) and aquatic organisms is widely recognised, affecting marine trophic levels from zooplankton (Heindler et al., 2017) to cetaceans (De Stephanis et al., 2013). In the Northern Hemisphere, densities of plastic items from micro (<5 mm diameter) to macroparticles (>20 mm) are present even in remote areas such as polar regions and the deep-sea, where they are coupled with longer time for complete degradation (Barnes et al., 2009). Phthalates are commonly used as emollients and softeners for these materials (Net et al., 2015), with an annual worldwide use of 8.4 million tonnes (ECPI 2020). These substances can represent up to 50% of the total weight of certain plastic products (Earls et al., 2003; Van Wezel et al., 2000). Phthalate additives are not chemically bound to the plastic matrix, resulting in a constant release from plastic products and a consequent ubiquitous presence in the environment via the processes of abrasion and leaching (Erythropel et al., 2014; Wittassek et al., 2011).

Di-2-ethylhexyl phthalate (DEHP) has been used as the most common softener of polyvinyl chloride (PVC) for years (Erythropel et al., 2014). Despite the evidence for its toxicity, it represents almost 40% of the global plasticiser market (ECPI 2020). The food chain is the most probable source for human exposure for long-chain phthalates such as DEHP, followed by skin route and inhalation (Schettler et al., 2006; Wittassek et al., 2011). Even though DEHP use is restricted in the European Union especially in toys and childcare articles, its levels are worldwide detected in freshwater (Li et al, 2016; Liu et al., 2014; Teil et al., 2007; Yu et al., 2009) and marine systems (Hermabessiere et al., 2017) at variable concentrations up to 0.6 µg/l in coastal waters (Sánchez-Avila et al., 2012) to over 50 µg/l in estuaries (Tan, 1995) and even 71.7 µg/l in Tunisian marine waters (Jebara et al., 2021). Presence of 30-5030 pg/l was even found in remote environments such as Arctic waters (Xie et al., 2007). The disruptive effect of DEHP, even at levels as low as 0.02 µg/l, is widely recognised by several studies that investigated the consequences in marine, brackish and freshwater species (Carnevali et al., 2010, Kim et al., 2002; Lu et al., 2013; Zanotelli et al., 2010) as well as terrestrial mammals (Kalo et al., 2015; Mu et al., 2015). At the molecular level, DEHP and similar endocrine disruptors are known to have a negative effect on lysosomal enlargement and membrane stability (Marigómez and Baybay-Villacorta 2003; Canesi et al., 2004), antioxidant and peroxisomal enzyme activities (Cancio et al., 1998; Orbea et al., 2002), lipid homeostasis

(Balbi et al., 2017) and shell formation (Balbi et al., 2016) in *Mytilus* spp. at levels of 10 - 500 µg/l.

Alongside plastic pollution, climate change is an emerging environmental cause of concern. In 2014, the fifth assessment report (AR5) from the Intergovernmental Panel on Climate Change (IPCC) communicated a linear increase of CO_2 emissions and a consequent rise of average temperatures around the globe. Moreover, in the special report of 2018, the scientific committee of the United Nations expressed its concern regarding the rising global temperature if no policies of reducing CO_2 emissions are promptly undertaken (IPCC, 2014, 2018). Several contaminants show a seasonal presence in water bodies, frequently due to temperature variations (Archana et al., 2017; Comber et al., 2020; Yang et al., 2017). High temperature can increase the rapidity of DEHP leaching from medical PVC infusion equipment both in dynamic systems and after static contact (Rose et al., 2012). In male molluscs, thermal stress was previously reported affecting *Mytilus galloprovincialis* sperm quality (Boni et al., 2016) and *Crassostrea virginica* testicular functions (Nash and Rahman 2019). In addition, survival, reproduction and growth of four freshwater mussels (*Amblema plicata*, *Elliptio complanata*, *Fusconaia flava* and *Lampsilis cardium*) were hypothesised to be affected by higher temperature by altering the reserves of metabolic energy (Ganser et al., 2015).

Biomarkers are used to assess whether organisms have been exposed to stressors and for determining the magnitude of their responses (Cajaraville et al., 2000). Contaminants and environmental factors such as temperature, air exposure, salinity and water pH could act synergistically to weaken defence and regulation mechanisms (Bodin et al., 2004; Viarengo et al., 1995). As an example, combinations of seawater acidification, high temperature and cadmium exposure had different effects on antioxidant levels and immune response of *M. galloprovincialis* (Nardi et al., 2017). In aerobic organisms, pro-oxidant stressors can promote overproduction of reactive oxygen species (ROS) that can lead to oxidative stress such as DNA damage, lipid peroxidation and enzyme inhibition if not counterbalanced by an adequate detoxification response (Abele and Puntarulo, 2004; Regoli and Giuliani, 2014).

Alongside abiotic factors, sex and sexual maturity may affect both contaminant uptake and elimination, and biomarker levels and activities (Blanco-Rayón et al. 2020; Burger et al., 2007; Damiens et al., 2004; McClellan-Green et al., 2007; Wilhelm Filho 2001). For example, common biomarkers of stress such as antioxidant and peroxisomal enzymes undergo seasonal variation during the annual reproductive cycle of *M. galloprovincialis* populations (Bocchetti and Regoli, 2006; Jarque et al., 2014), or when exposed to multiple stressors in different seasons (Nardi et al., 2018). Additionally, natural differences in basal antioxidant levels between males and females could favour one sex over the other when coping with stressful environments (Gismondi et al., 2012; Grilo et al., 2018). Considering this, assessing sex and reproductive status could be a valuable asset in interpreting toxicology results.

Recently, the effect of plastic contamination on mussels has been investigated for short- and long-term exposures (Avio et al., 2015; Bråte et al., 2018; Paul-Pont et al., 2016; Pittura et al., 2018). In *Mytilus* spp., the exposure to nano- and microplastic particles in combination with the anticonvulsant drug carbamazepine decreased the heat shock protein 70 gene expression in gills (Brandts et al., 2018), and had an effect on catalase gene expression in combination with the polycyclic aromatic hydrocarbon fluoranthene in gills and digestive gland (Paul-Pont et al., 2016). However, little is still known about the combined effect of plastic additives and climate change-related stressors on biological responses of *Mytilus* spp..

This study aims to investigate the responses of blue mussels to DEHP exposure and increased temperature, separately and in combination, through a factorial design. It is relevant for understanding the effect of multiple stressors on sentinel species for plastic pollution in the context of global warming. For this reason, two temperatures and two different concentrations of DEHP were chosen. Responses at the level of gene expression were studied for superoxide

dismutase, catalase and heat shock protein 70 (*sod*, *cat* and *hsp70*), as representatives of the mussel stress response. Genes coding for SOD and CAT were chosen as these are antioxidant enzymes that can counteract the damaging effect of oxyradicals (Regoli and Giuliani 2014). SOD converts ROS superoxide anion radical O_2^- into oxygen and hydrogen peroxide (H_2O_2), which is reduced by CAT. H_2O_2 increases under thermal stress (Abele et al., 2007) and causes harm to other cellular reactions if not removed (Lesser 2006). HSP70 was chosen as a highly conserved and versatile member of the HSP family, particularly due to its responsiveness to environmental perturbation (Encomio and Chu, 2005; Lewis et al., 1999). HSP70 is linked not only to changes in temperature, but also with protein metabolism under stress conditions, membrane translocation, regulatory processes and misfolded protein sequestration (Feder and Hofmann 1999; Fink 1999; Franzellitti and Fabbri 2005; Hartl 1996). Additionally, genes for estrogen-related receptor (*MeER1*) and estrogen receptor (*MeER2*) were chosen as reprotoxicity parameters because of their possible involvement in estrogen signalling and the reproductive cycle in *Mytilus* species (Ciocan et al., 2011; Nagasawa et al., 2015) and their different expressions at different stages of mussel gonadal maturation and exposure to estrogens (Agnese et al., 2019; Ciocan et al., 2010).

2. Materials and Methods

2.1 Experimental design

Adult blue mussels (n = 180; length mean ± standard deviation = 5.4 cm ± 0.6 cm) were collected at low tide from the intertidal zone at Filey Bay, North Yorkshire, UK (54° 13´ longitude; 0° 16´ latitude, T_{water} = 11°C; pH = 8.07) in November 2018 and transported to the aquarium facilities of the University of Hull. Environmental background DEHP contamination from the collection site was considered low, in light of the Filey area being adjacent to a monitored marine protected zone and the mussels undergoing an acclimation period to the lab conditions that served as a depuration period. In blue mussels, sexual maturity is not related to increased size (Seed, 1969). For this reason, mussels were randomly divided into six different 4-litre continuously aerated glass tanks for each treatment and kept for acclimation for 12 days in artificial saltwater (Premium REEF-Salt, Tropical Marine Centre, Chorleywood, UK) under laboratory conditions. These were a salinity of 35‰ and a pH of 8.1 units, a photoperiod of light:dark = 10h:14h and water temperature of 11°C, which reflects the average winter water temperature in Filey Bay (low temperature treatments CTRL), and 14°C (high temperature treatments HIGH T). The total increase of +3°C for the high-temperature treatment (T = 14°C, HIGH T) reflects the current average summer temperatures in Filey Bay and was chosen in light of the mean temperature range of the global warming scenarios projected for the end of the century (IPCC, 2014). For the high temperature treatment, temperature was progressively raised by 1°C during the first three days of acclimation by adjusting ambient air temperature in a climate-controlled room, as high temperature is an important factor in controlling the reproductive cycle (Bayne 1976a, b) and in order to avoid an immediate temperature-induced shock. After the acclimation period, mussels of low and high temperature treatments were additionally exposed for seven days to two different concentrations of DEHP (0.5 LOW DEHP and 50 µg/l HIGH DEHP). DEHP (≥ 99.5% purity) was obtained by Sigma Aldrich, Gillingham (UK) and dissolved in ethanol, in order to prepare a stock solution of 1 mg/ml. DEHP was dosed every other day. The two DEHP concentrations of 0.5 and 50 µg/l were chosen from the literature, aligned to the DEHP levels detected in natural environments, where the minimum and maximum average levels for marine environments were found to be 0.145 µg/l (Sánchez-Avila et al., 2012) and 71.7 µg/l (Jebara et al., 2021). A seven-day exposure to DEHP was chosen considering that phthalates such as DEHP are not expected to be highly persistent

compounds in many environments (Staples et al. 1997) and estimated environmental persistence values for DEHP are approximately 1 day for atmospheric half-life, and 0.35 - 3.5 days for surface water and sediment half-life in aerobic conditions (Peterson and Staples 2003). During the exposure period, animals were not fed, and water was changed every second day with DEHP dosed right after (i.e. days 1, 3 and 5). Temperatures were kept at 11 °C or 14 °C, respectively. Together with CTRL and HIGH T treatments, this yielded a total of six treatments. Salinity remained constant at 35 ± 1 psu over the course of the experiment in all conditions. Temperature, pH and salinity were measured daily (Supplementary Table 1) with a digital thermometer (model ama-digit ad 15 th, Amarell™ Thermometer, Kreuzwertheim, Germany), an Accumet™ portable pH-meter (Thermo Fisher Scientific, Loughborough, UK) and a digital seawater refractometer (Hanna Instruments®, Woonsocket, USA). After seven days of exposure, mussels were sampled (under unlicensed animal ethics approval University of Hull #U080/FEC_2021_11) and gonadal tissues were collected for molecular and histological analyses. For molecular analysis, *ca.* 1.0 cm^2 of gonad tissue was dissected and immersed in 1 ml RNAlater® Stabilisation Solution (Thermo Fisher Scientific, Loughborough, UK) and stored at -80 °C until extraction. Approximately the same quantity of tissues was stored in 1 ml neutral-buffered 10% formalin solution (Sigma Aldrich, Gillingham, UK) at room temperature for histological analysis.

2.2 Histological analysis

Gonad samples were washed with phosphate-buffered saline (PBS), dehydrated with increasing ethanol concentrations (70, 90, 100%), cleared with Histoclear II® (National Diagnostics, Atlanta, USA) and embedded in paraffin wax. Tissue sections (10 µm) of gonads were cut on a manual microtome (Thermo Fisher Scientific, Loughborough, UK) and stained using haematoxylin and eosin solutions (Sigma Aldrich, Schnelldorf, Germany). Prior to microscopic analysis, microscope slides were coded, in order to conduct a blind observation. Males and females were identified under a light microscope, and the following gametogenesis stages were blindly assessed, following the stage descriptions reported by Seed (1969), as follows: (i) resting or spent gonad (Fig. 1A); (ii) development stage 1 (Fig. 1B and 2A); (iii) development stage 3 (Fig. 1C and 2B); iv) development stage 5: mature stage (Fig. 1D and 2C); (v) spawning stage 3 (Fig. 1E and 2D); (vi) spawning stage 1 (Fig. 1F).
Each stage was categorised by a maturity factor (MF): (i) MF = 1 for resting or spent gonad; (ii) MF = 2, developing gonads (stage 1 and 3); (iii) MF = 3, mature gonads; (iv) MF = 4, spawning gonads (stage 1 and 3). Then, the sexual maturity index (SMI) was calculated according to the equation established by Siah et al., (2003): $SMI = \Sigma$ *(proportion of each stage * maturity factor)*. Further details are given in the Supplementary methods.

2.3 Gene expression

Samples per treatment and sex (*approx.* 10 mg of gonad tissue, n = 96) were selected randomly and blindly coded for the analysis, and total RNA was extracted from gonadal tissues using the High Pure RNA Tissue Kit (Roche Applied Science, Burgess Hill, UK), including a 15 min DNase I treatment at 25°C. Eventually, 8 female gonads and 8 male gonads for each treatment were processed for total RNA extraction. cDNA templates were synthesised using 200 units of Invitrogen™ SuperScript™ II Reverse Transcriptase (Fisher Scientific, Loughborough, UK). Sample species were identified by PCR and agarose gel electrophoresis for the non-repetitive region of the *Mytilus foot protein 1 (mfp-1)* gene using the primer sets published in Inoue et al., (1995). Further details are given in the Supplementary methods.
Primer sequences were taken from the literature: *elongation factor-1 alpha (EF1α)* (GenBank accession no. **AF063420**), *18SrRNA (Me18S)* (**L33448**) and *28SrRNA (Me28S)* (**Z29550**) from

Ciocan et al., (2011); *catalase (cat)* (**AY580271**) from Lacroix et al., (2014); *estrogen receptor 2 (MeER2)* (**AB257133**) from Puinean et al., (2006). Additionally, new primers were designed using Primer3 (http://primer3.ut.ee/) from published sequences (*superoxide dismutase (sod)* (**AJ581746**)*; heat shock protein 70 (hsp70)* (**AF172607**)*; estrogen receptor 1 (MeER1)* (**AB257132**). Primer details are provided in Supplementary Table 2. Only primer efficiencies between 90 and 110% were accepted (Supplementary Table 2), in accordance with the MIQE guidelines (Bustin et al., 2009). *Me18S*, *Me28S* and *EF1α* were chosen as they represent suitable reference genes during mussel gametogenesis and exogenous estrogen exposures (Cubero-Leon et al., 2012). *Me18S* and *Me28S* genes were chosen for normalisation of the final dataset using the $2^{-\Delta Ct}$ method (Schmittgen and Livak 2008), being considered the most stable combination by RefFinder software and Kruskal-Wallis test (*Me18S* KW-H = 4.956, p = 0.4213; *Me28S* KW-H = 5.464, p = 0.3619; *EF1α* KW-H = 4.4484, p = 0.4858). qPCR reactions were performed on a CFX96 Real Time PCR Detection System (Bio- Rad, Hemel Hempstead, UK) using 10 µL of PrecisionPlus qPCR Master Mix premixed with SYBR Green (PrimerDesign, Eastleigh, UK), 7.5 µL molecular-grade water, 1 µL of each primer, and 0.5 µL cDNA. Final primer concentrations are given in Supplementary Table 2. Sample dilutions (1:10) were used in combination with *Me18S* and *EF1α* primers. Thermal cycling was as follows: 95°C for 2 min, 40 cycles of 95°C for 10 sec, 60°C for 1 min and 72° C for 1 min. Template-negative reactions were included alongside samples. Primer specificity and absence of secondary product formations were demonstrated by the melt peaks at the conclusion of the reactions. Further details are given in the Supplementary methods.

2.4 Statistical analysis

The data from histological analysis of gametogenesis stage was analysed with ordinal logistic regression, to predict the dependent variable "gametogenesis stage", assuming "DEHP", "temperature" and "sex" as independent variables. Model uncertainty was assessed by comparing ΔAICc values and Akaike weights in which the lowest values for ΔAICc indicate second best to least parsimonious models of the set (Table 1). Model selection was carried out in RStudio with the *AICcmodavg* package (Mazerolle 2016) in R 3.6.2 (CRAN). Ordinal logistic regression was carried out using the *polr* function (*MASS* package, Venables and Ripley 2002), calculating the p error probability comparing the t-value against the standard normal distribution (Table 2). The proportional odds assumption (test of Parallel Lines) was tested using the *ordinal* package (Christensen 2019).

Regarding the effect of different treatments on the mRNA expressions of each gene, the non-parametric Scheirer-Ray-Hare (*rcompanion* package, Mangiafico 2017) test was used on the $2^{-\Delta Ct}$ values, after verifying non-normal distribution (Shapiro-Wilk test) and homogeneity of variances (Levene's test). Dunn's multiple comparison test with Benjamini & Hochberg p-adjustment was used for comparisons between groups (Benjamini and Hochberg 1995). Possible outliers were identified by Grubbs' test (Grubbs 1969) and outlier values beyond 99% of the range of the characterised dataset distribution were rejected (Burns et al., 2005; ISO 5725-2 2019). Additional permutation multivariate analysis of variance (PERMANOVA, Anderson 2014) was used in Rstudio (*vegan* package, Oksanen et al., 2013), for testing the effects of the exposure conditions on the $2^{-\Delta\Delta Ct}$ values of the stress-related mRNA expression (*sod*, *cat* and *hsp70*) and estrogen receptor-like mRNA expression (*MeER1* and *MeER2*) using Bray-Curtis distance and 9999 permutations. Pairwise multilevel comparison with Benjamini & Hochberg p-adjustment was used to compare different groups. Statistical significance was set to $p < 0.05$. All graphs were created using MATLAB R2019b. Further details are given in the Supplementary methods.

3. Results

3.1 Histology results to determine gametogenesis stages

In males, control groups displayed percentages of developing and spawning stages in line with the natural cycle of *M. edulis* during the winter season. A similar trend is noticeable in the treatments exposed to DEHP (LOW DEHP and HIGH DEHP) at control temperature. On the contrary, all the high-temperature treatments (HIGH T, LOW DEHP HIGH T and HIGH DEHP HIGH T) exhibited higher percentages of spawning gonads, with a consequent increase of the SMIs. In contrast, female mussels displayed similar gametogenesis stages and SMIs for all the treatments (Fig. 1, 2 and 3).

The most parsimonious ordered logistic regression model (TEMP*SEX) showed that the difference in temperature had a significant effect on the transition from development to spawning stage (p = 0.005). Moreover, the model demonstrated that males and females displayed significant differences in the proportion of developing and spawning stages (p = 0.05) with a pronounced increase in combination with temperature (p = 0.007). Conversely, DEHP treatments did not significantly affect gametogenesis stages in either males or females.

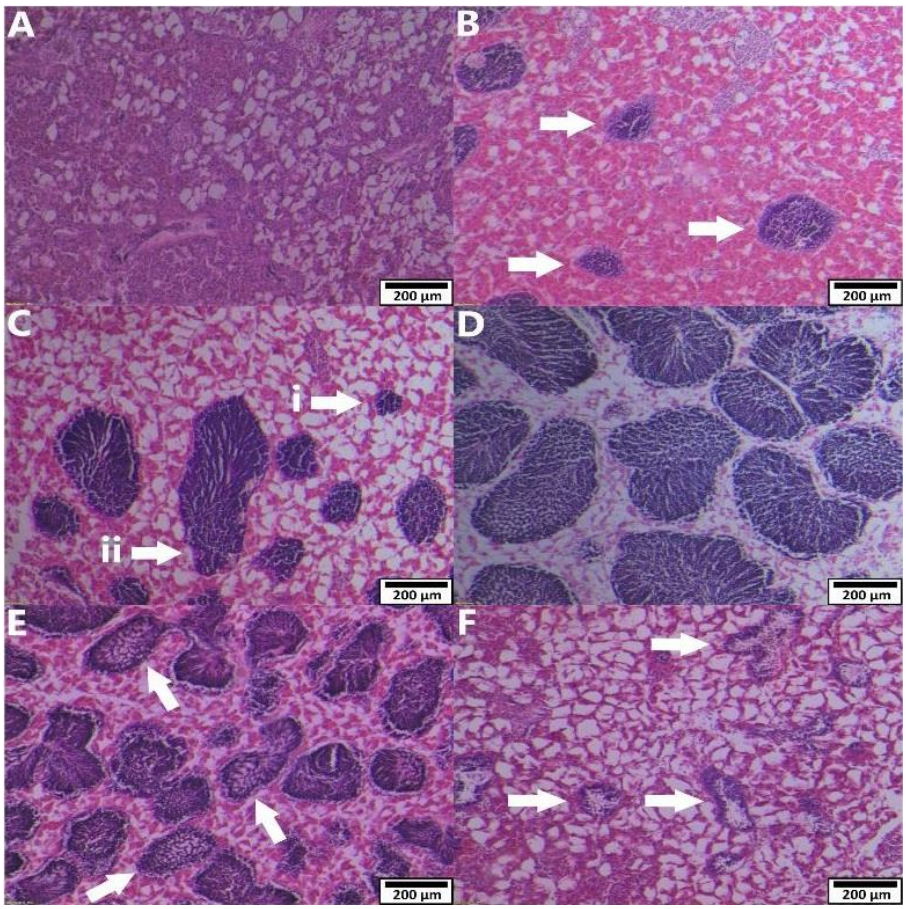

Fig. 1 Gametogenesis stages of 10µm gonadal tissue sections stained with haematoxylin and eosin of males. Resting stage (undetermined sex, A, at 10x magnification), follicles at development 1 (B, 10x), development 3 with different follicle sizes (i and ii) (C, 10x), mature stage (D, 10x), empty follicles at spawning 3 (E, 10x) and spawning 1 (F, 10x). Scale bars represent 200 µm. Images were modified for brightness and contrast

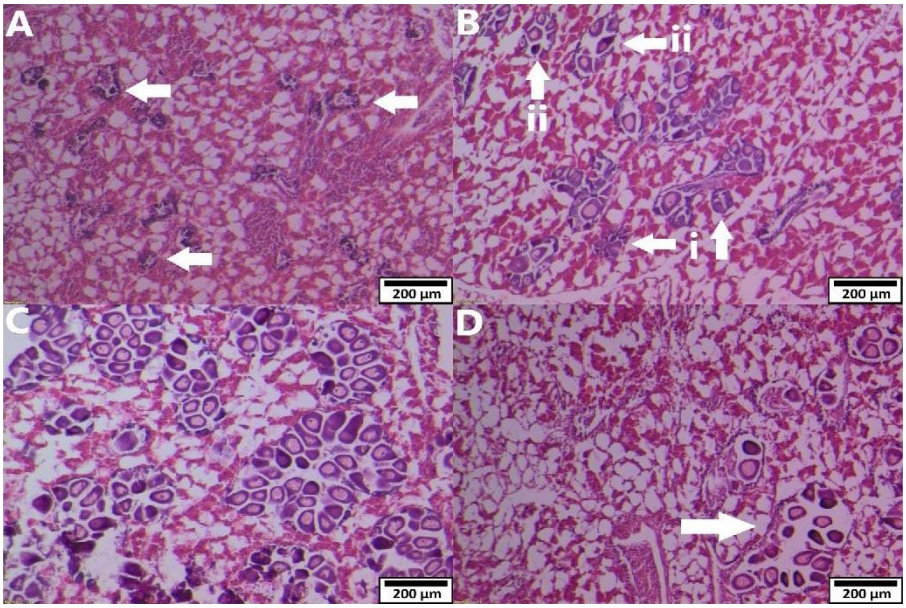

Fig. 2 Gametogenesis stages of 10µm gonadal tissue sections stained with haematoxylin and eosin of females. Irregular follicles and small oocytes in development 1 (A, 10x), early (i) and mature (ii) gametes in development 3 (B, 10x), mature stage (C, 10x) and empty follicles in spawning 3 (D, 10x). Scale bars represent 200 µm. Images were modified for brightness and contrast

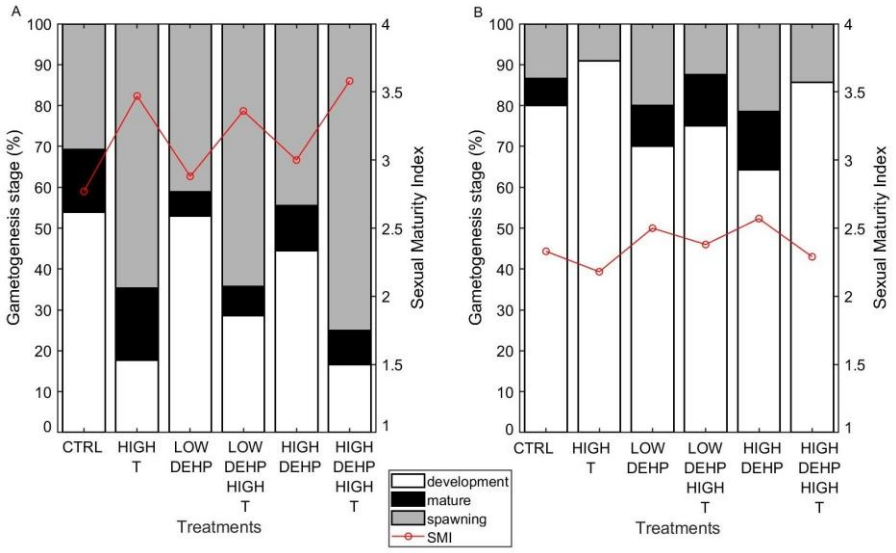

Fig. 3 Effects of temperature and DEHP on gametogenesis stages. Percentage of each stage and sexual maturity index (SMI) of males (A, left) and females (B, right) in CTRL (n = 13 (A), 15 (B)), HIGH T (n = 17 (A), 11 (B)), LOW DEHP (n = 17 (A), 10 (B)), LOW DEHP HIGH T(n = 14 (A), 8 (B)), HIGH DEHP (n = 9 (A), 14 (B)) and HIGH DEHP HIGH T (n = 12 (A), 14 (B))

Table 1 Model classification, number of estimated parameters (K) for each model, Akaike Information Criterion (AICc), delta AIC (ΔAIC), Akaike weights (AICcWT), cumulative Akaike weights (CumWT), log-likelihood of each model (LL) for the three independent variables (+) temperature (TEMP), DEHP concentration (DEHP) and sex (SEX) and their interactions (*) on gametogenesis stages

model	K	AICc	Δ AIC	AICcWT	Cum WT	LL
TEMP*SEX	5	251.13	0.00	0.8175	0.8175	-120.499
TEMP+SEX	4	256.71	5.58	0.0501	0.8677	-124.313
SEX	3	257.04	5.91	0.0425	0.9101	-125.496
TEMP+DEHP+SEX	5	357.79	6.66	0.0293	0.9394	-123.828
TEMP*DEHP*SEX	9	258.02	6.89	0.0261	0.9655	-119.812
DEHP+SEX	4	258.09	6.96	0.0251	0.9906	-125.003

DEHP*SEX	5	260.04	8.94	0.0094	1	-124.969

Table 2 Results of ordinal logistic regression for the best model of treatments (TEMP*SEX). Estimated value, standard error, *t*-value and *p* for the independent variables temperature (TEMP, 11 and 14 °C), and sex (SEX, males and females) and their interactions

Variable	Value	Std. Error	*t*-value	*p*
TEMP	1.266	0.446	2.837	0.005
SEX	-0.933	0.469	-1.991	0.046
TEMP:SEX	-2.008	0.747	-2.687	0.007

3.2 Expression of stress-related and estrogen receptor-like genes

Regarding gene expression in male mussels, temperature and DEHP exposure had no effect on the expression of *sod* and *MeER1* ($p > 0.05$) (Supplementary Fig. 1 and 4). In contrast, *cat* mRNA expression was significantly increased by temperature (SRH $p = 0.041$) and the combined effect of temperature and DEHP (SRH $p = 0.040$) (Supplementary Fig. 2). *hsp70* mRNA expression was marginally (but not significantly) modulated by DEHP exposure (SRH $p = 0.079$) and a slight but also non-significant effect of temperature was observed (SRH $p = 0.102$) (Supplementary Fig. 3). *MeER2* expression was significantly increased in males in the high temperature treatments (SRH $p = 0.011$) (Supplementary Fig. 5).

In female mussels, *MeER1* mRNA expression was significantly influenced by the DEHP exposure (SRH $p = 0.041$) (Supplementary Fig. 9). Neither temperature nor DEHP exposure had an effect on the expression of *sod*, *cat*, *hsp70* and *MeER2* ($p > 0.05$) (Supplementary Fig. 6, 7, 8 and 10). Details of mRNA expression levels of each gene are provided in Supplementary Tables 3 and 4.

Considering the overall expression of stress-related genes in males, PERMANOVA analysis underlined a significant effect of temperature on increased mRNA expressions of *sod*, *cat* and *hsp70* ($p = 0.014$) and a slight but non-significant effect of DEHP in lowering the expression of these genes ($p = 0.060$) (Fig. 4 left). Moreover, temperature increased the expression of *MeER1* and *MeER2* in males ($p = 0.005$), but low and high DEHP treatments had opposite effects on gene expression (Fig. 5 left). Regarding females, no significant effect of DEHP and temperature was noticeable on stress response-related gene expression (Fig. 4 right), but DEHP exposure had a significant effect on lowering gene expression related to the estrogen receptor-like response ($p = 0.049$) (Fig. 5 right). In both sexes and gene groups, the LOW DEHP treatment caused a decrease in gene expression, which was not mirrored by the HIGH DEHP treatment. Expression of stress-related genes in LOW DEHP treatments differed between males and females (Table 3). Expression in the HIGH DEHP treatment in both gene groups was significantly different between males and females (Table 3, 4).

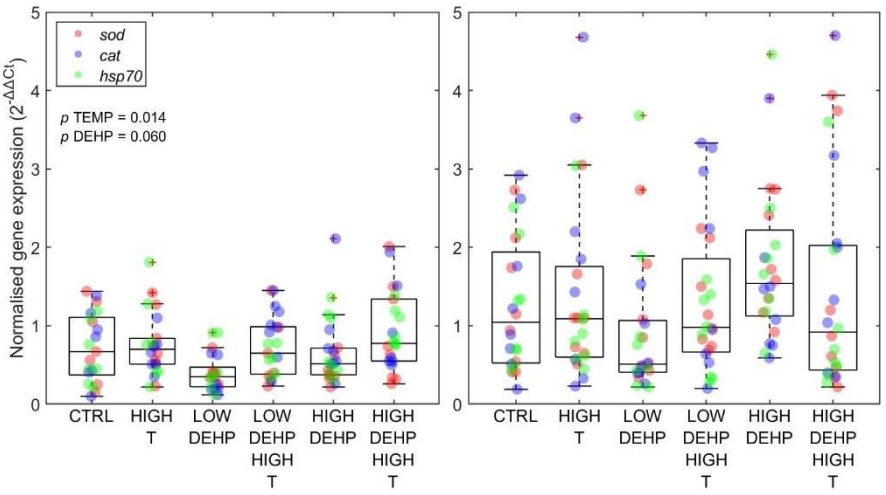

Fig. 4 left) Stress-related (*sod*, *cat*, *hsp70*) gene expression in males, n = 6 to 8. **right)** Stress-related (*sod*, *cat*, *hsp70)* mRNA expression in females, n = 7 to 8. Abbreviations are control (CTRL), high temperature (HIGH T), low DEHP concentration (LOW DEHP), low DEHP at high temperature (LOW DEHP HIGH T), high DEHP concentration (HIGH DEHP) and high DEHP at high temperature (HIGH DEHP HIGH T). PERMANOVA error probabilities are annotated. Individual gene expression plots are shown in Supplementary Figures 1 - 3 and 6 - 8

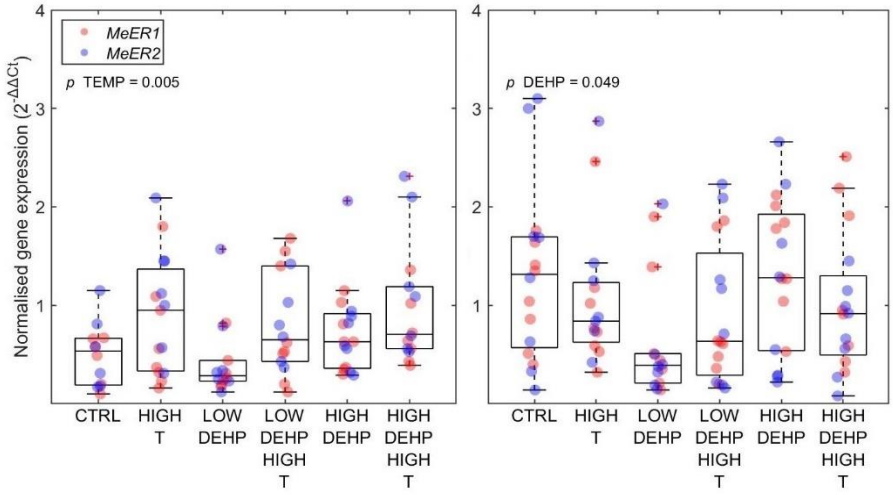

Fig. 5 left) Estrogen receptor-like (*MeER1*, *MeER2*) gene expression in males, n = 6 to 8. **right)** Estrogen receptor-like (*MeER1*, *MeER2*) mRNA expression in females, n = 7 to 8. Abbreviations are control (CTRL), high temperature (HIGH T), low DEHP concentration (LOW DEHP), low DEHP at high temperature (LOW DEHP HIGH T), high DEHP concentration (HIGH DEHP) and high DEHP at high temperature (HIGH DEHP HIGH T). PERMANOVA error probabilities are annotated. Individual gene expression plots are shown in Supplementary Figures 4 - 5 and 9 - 10

Table 3 Pairwise multilevel comparisons of the stress response (*sod*, *cat*, *hsp70*) between males and females in the same treatments

Treatment males	**Treatment females**	***p***
CTRL	CTRL	0.138
HIGH T	HIGH T	0.131
LOW DEHP	*LOW DEHP*	*0.046*
LOW DEHP HIGH T	LOW DEHP HIGH T	0.108
HIGH DEHP	*HIGH DEHP*	*0.001*
HIGH DEHP HIGH T	HIGH DEHP HIGH T	0.454

Table 4 Pairwise multilevel comparisons of the estrogen receptor-like response (*MeER1*, *MeER2*) between males and females in the same treatments

Treatment males	Treatment females	*p*
CTRL	CTRL	0.056
HIGH T	HIGH T	0.543
LOW DEHP	LOW DEHP	0.525
LOW DEHP HIGH T	LOW DEHP HIGH T	0.618
HIGH DEHP	*HIGH DEHP*	*0.020*
HIGH DEHP HIGH T	HIGH DEHP HIGH T	0.438

4. Discussion

Even though the consequences of global warming and plastic pollution have been investigated in several species, the biological responses under combined stressors related to these events are just now emerging. In this study, we show that temperature and DEHP exposure influence males and females differently, with the potential to disrupt the synchronicity of breeding.

Control individuals exhibited gametogenesis stages in line with values previously reported from the Filey Beach area during wintertime (Chapman et al., 2017; Seed 1969), with some individuals having spawning gonads alongside a higher percentage of still developing mussels. The normal gametogenesis cycle of North Yorkshire mussels starts in late autumn with declining temperatures, until maturation and spawning in spring and summer (Bayne 1976a,b), but high temperature and other environmental stress cues are shown to induce attenuated seasonality in bivalve gametogenesis cycle and increased or advanced spawning activity instead of only one peak per season (Bayne 1976b; Petes et al., 2008; Philippart et al., 2003; Sreedevi et al., 2014). An accelerated gametogenesis cycle might not provide the time necessary for storing energy (e.g. glycogen) during resting periods, causing deleterious consequences in offspring quality and impairment of reproductive capacity (Fearman et al., 2009).

Here, males and females presented an inverted SMI trend in the high temperature treatments, showing advanced development in males, but a delay in females. Females can naturally display a slight asynchrony with males with no repercussions on the reproductive success (Azpeitia et al., 2017; Seed 1969). However, if periods of thermal stress accelerate only the male cycle, this asynchrony would get further pronounced. Both an increase in the release of gametes through continuous spawning, and a reduction of fertilisation events through increased asynchrony in gametogenesis are possible outcomes. Both mechanisms, however, could alter the temporal pattern of juvenile densities, which might impact coastal population dynamics of mussels.

In the present study, cDNA samples were identified as blue mussel complex, due to the natural admixture and genomic overlaps between *Mytilus* spp. species that commonly occur in the Northern Hemisphere caused by incomplete reproductive isolation (Simon et al., 2020).

While overall stress response (i.e., *sod*, *cat*, *hsp70* gene expression) of males was significantly induced by temperature but not DEHP exposures, no adverse effects of treatments on these oxidative and molecular chaperone stress response markers were observed in females. Estrogen receptor-like responses (i.e., *MeER1* and *MeER2* gene expression) were affected by temperature in males and DEHP exposure in females, respectively.

An increase in temperature accelerates metabolic rate, mitochondrial respiration and production of ROS, raising the energy demand with a consequent oxygen deficit (Abele 2002; Lushchak 2011). The resulting stress responses at the cellular level should include the stimulation of essential antioxidant defences such as CAT, a key enzymatic catalyst for reduction of the ROS hydrogen peroxide (H_2O_2) to water (Halliwell and Gutteridge 2015; Regoli and Giuliani 2014). Rising CAT levels by increasing temperature have already been

demonstrated in different phyla, including molluscs (Abele et al., 1998; Hu et al., 2015; Rahman et al., 2019; Verlecar et al., 2007). Interestingly, in this study, high temperature did not only have a significant effect on *cat* expression in males but seemed to accentuate the toxic effect of DEHP as well. A significant effect was found for both stressors combined, especially in the low concentration treatment. Similarly, hepatocytic CAT in zebrafish *Danio rerio* exposed to the aquatic endocrine disruptor levonorgestrel combined with high temperature showed increased levels under low concentrations while control and high concentration treatments exhibited similar values (Cardoso et al., 2019).

It is worth noting that while *cat* expression is influenced by environmental stressors, these did not elicit a significant response on *sod* levels in either sex. Differences in the activities of CAT and SOD were already observed in previous experiments involving mussels at different days of exposure to heavy metals and endocrine disruptors (Gonzalez-Rey and Bebianno 2013; Orbea et al., 2002), showing in some cases even an inverted trend (Monteiro et al., 2019), which underlines the lack of knowledge about inhibition patterns in the antioxidant system of mussels exposed to different or prolonged stressors.

Regarding *hsp70* expression in males, even though neither the effect of temperature nor of DEHP was significant, a noticeable trend of both stressors to affect *hsp70* expression in males was observed. HSP70s play a crucial role in repairing partially denatured proteins and in the cellular protection from stress-induced damage, protein folding and translocation (Feder and Hofmann 1999; Fink 1999) and they can be induced by both physical and chemical stressors (Sanders 1993). Sex dissimilarities in HSP levels were already noticed in species such as *Pachygrapsus marmoratus* and *Daphnia magna* crustaceans (Madeira et al., 2012; Mikulski et al., 2011), possibly related to hormonal regulation, reproductive fitness or habitat adaptation strategies. The slight increase of *hsp70* expression to higher temperature in males could be explained with an attenuated heat-shock response under laboratory acclimation (Roberts et al., 1997), or the recent thermal history of the organism (Buckley et al., 2001). Due to the high-energy cost required for the HSP synthesis, a heat shock response that is mitigated over periods of persistent stress, might allow to conserve energy reserves for other fitness-relevant processes (Tomanek and Somero 1999; Troschinski et al., 2014). On the other hand, as remarked by Franzellitti and Fabbri (2005), the partial sequence for *M. edulis hsp70* used in this work could encode for a constitutive HSP70 isoform that is more associated with prolonged stress exposure instead of short-term responses.

In contrast to males, females did not show any significant differences in the expression of *sod, cat, hsp70*. Sex and sexual stage are known to influence physiological and morphological traits differently, including the stress response, and lead to a disparate uptake and effects of contaminants (Blanco-Rayón et al., 2020; Burger 2007; Jarque et al., 2014; Louis et al., 2021). In *M. edulis,* differences between sexes were noted in regard to gonadal seasonal expression of clock-associated genes in response to environmental cues (Chapman et al., 2017; Chapman et al., 2020), and polar mantle metabolite concentrations after spawning (Hines et al., 2007). Moreover, sex dissimilarities in protein profiles of the zebra mussel *Dreissena polymorpha* gills were observed after exposure to different concentrations of the polycyclic aromatic hydrocarbon (PAH) benzo(α)pyrene (Riva et al., 2011). Sex-associated differences in energy allocation and metabolic status could influence the vulnerability of either sex to thermal stress, which could alter the composition or abundance of populations (Bedulina et al., 2017). Our results show a higher sensitivity of males under environmental stressors, which in turn could suggest a more resilient status of developing females under prolonged stress conditions.

In Nagasawa et al., (2015), two different estrogen receptor-like genes were found expressed in both ovaries and testes of *M edulis* and *M. galloprovincialis* (estrogen receptor ER (*MeER2*) and estrogen-related receptor ERR (*MeER1*)), suggesting their involvement in reproduction (Croll and Wang 2007). Their expression was recently found to differ between maturation

stages during the ovarian cycle of *M. galloprovincialis* (Agnese et al., 2019), but the exact nature of their involvement in the estrogen signalling and the reproductive cycle is still unknown. Surprisingly, our study highlighted a temperature effect on *MeER2* expression in males. Males in higher temperature treatments displaying advanced spawning stages may constitute a causal link to elevated *MeER2* levels. A similar effect of temperature on *MeER2* upregulation was recently observed by Koagouw and Ciocan (2018). Smolarz et al. (2018) suggested that steroids such as estrogens and androgens might be active modulators of only the final stage of the gametogenesis cycle (i.e. spawning), as they appear to be more associated with environmental cues such as water temperature. The presence of endogenous sex steroid hormones has been observed in invertebrates (Köhler et al., 2007; Reis-Henriques et al., 1990; Stefano et al., 2003), which might suggest that molluscs could share an ancestral estrogen-related signalling mechanism with vertebrates (Eick and Thornton 2011; Thornton et al., 2003). However, their functions and regulation in *Mytilus* spp. remain unclear (Puinean and Rotchell 2006). *MeER2* expression in *M. edulis* in their natural environment varied in mature gonads in different years (Ciocan et al., 2010) suggesting that annual and seasonal environmental cues could lead to nuances in the gametogenesis status and the related estrogen-like responses. In contrast to the temperature effect, the exposure to environmentally relevant concentrations of DEHP (both 0.5 and 50 µg/l) did not elicit an estrogenic effect on males. Similarly, no effects were found in male ricefish *Oryzias latipes* exposed to concentration up to 50 µg/l (Kim 2003), but significantly higher concentrations of 100 and 500 µg/l caused an increase in estradiol levels and reduced number of spermatozoa in *Oryzias melastigma* males (Ye et al., 2014). Our results suggest no reprotoxicity effect of DEHP on gametogenesis timing in male blue mussels at environmentally relevant concentrations, but other adverse effects such as germ cell toxicity cannot be excluded at this time.

In this study, DEHP induced an effect on females' *MeER1* expression. Considering *MeER1* levels are lower in the low-concentration groups compared to both control and high DEHP treatments, a dose-response does not appear to be present. Here, we found a higher expression of *MeER1* in response to 50 µg/l DEHP at control temperature. Molluscs seem more sensitive to exposure to plasticisers in water at concentrations in the order of magnitude of micrograms per litre (Oehlmann et al., 2009), and endocrine active chemicals can follow a nonmonotonic dose-response curve, stimulating a response at low concentration and inhibiting it at higher levels (Conolly and Lutz 2004; Do et al., 2012; Li et al., 2007; Vandenberg et al., 2012). Furthermore, it was recently described that low doses of DEHP can impair the meiotic processes in *Caenorhabditis elegans* nematodes, through considerable morphological defects of chromosomes in oocytes and impaired embryogenesis (Cuenca et al., 2020). Therefore, in light of the effect of DEHP on the female estrogen receptor-like responses observed in this study, we cannot exclude an impairment of their reproductive cycle that could affect not only the egg maturation but also larval development.

5. Conclusion

In conclusion, a sex-dependent response is observable after the exposure to high temperature (14°C) and two environmentally relevant concentrations of the plasticiser DEHP (0.5 µg/l and 50 µg/l), highlighting, as have other studies before ours (Banni et al., 2011; Chapman et al., 2017; Koagouw and Ciocan, 2019; Liu et al., 2017; Matozzo and Marin, 2010), the importance for sex identification in bivalve experiments. Male mussels were observed to be more sensitive to thermal stress, demonstrated by advanced gametogenesis, and an increase in both the expression of gametogenesis-related as well as antioxidant response-related genes (particularly *MeER2* and *cat*). On the other hand, females were affected by the presence of the plastic

additive, which affected *MeER1*, a gene putatively involved in the endocrine pathway. Recently, the closely related species *M. galloprovincialis* was revealed to be the first species in the animal kingdom possessing a pan-genomic architecture with 25% of genes subject to presence/absence variation, most of them possibly involved in stress response and survival systems (Gerdol et al., 2020). Considering this, further studies are needed to better understand dose-responses of bivalves to combined climate stressors and plastic additive exposure, whose reprotoxicity in sentinel species is still not fully established.

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Declaration of Competing Interest

The authors report no conflict or declarations of interest.

Availability of data and material (data transparency)

Additional methods are available as Supplementary Methods. Experimental treatments and measurements of experimental conditions can be found in Supplementary Table 1. Primer details can be found in Supplementary Table 2. Single gene expression values for males and females can be found in Supplementary Tables 3 and 4 and Supplementary Figures 1 - 10.

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