



Combined effects of climate change and environmentally relevant mixtures of endocrine disrupting compounds on the fitness and gonads' maturation dynamics of *Nucella lapillus* (Gastropoda)

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ABSTRACT

Coastal areas are affected by multiple stressors like climate change and endocrine disruptors (EDCs). In the laboratory, we investigated the combined effects of increased temperature and EDCs (drospirenone and mercury) on the fitness and gonads' maturation dynamics of the marine gastropod *Nucella lapillus* for 21 days.

Survival was negatively affected by all the stressors alone, while, in combination, a synergistic negative effect was observed.

Both chemicals, as single factors, did not cause any effect on the maturation stage of ovaries and testis. However, in the presence of a higher temperature, it was clear a delay in the maturation stage of the ovaries, but not in the testis, suggesting a higher negative impact of the stressors in females than in males.

In summary, drospirenone caused a low negative impact in aquatic species, like gastropods, but in combination with other EDCs and/or increased temperature can be a matter of concern.

1. Introduction

In the context of climate change, it is known that marine ecosystems have undergone constant changes. According to the Intergovernmental Panel on Climate Change (IPCC), it was estimated that, by 2100, the average global surface temperature will increase between 3.3 and 5.7 °C and the average surface temperature of the oceans will increase between 3 and 4 °C (Arias et al., 2021; Stocker, 2014), which will bring strong implications on the structure and functioning of coastal ecosystems (Arias et al., 2021; Wernberg et al., 2011). Temperature is one of the physicochemical variables, which plays an important role in the general functioning of aquatic communities (Brown et al., 2004). It is proved that global warming can alter feeding and growth rates (Miller, 2013) as well as the reproduction of many species (DeCourten and Brander, 2017), which will, ultimately, have cascading effects on all the marine communities (Miller, 2013).

Besides climate change, marine ecosystems are constantly being threatened by contaminants of anthropogenic origin, being the endocrine disruptor compounds (EDCs) one of the main priority topics for the

European Union (Peterson et al., 2007; EU Commission, 2011).

Endocrine disruptors mostly act as mimicking natural hormones, but some of them can antagonize the action or modify the synthesis, metabolism and transport of endogenous hormones, producing a range of developmental, reproductive, neurological, immune, or metabolic diseases in humans and wildlife (Khetan, 2014).

One of the types of EDCs with the greatest impact on the aquatic systems are the pharmaceutical compounds, being one of the most critical steroid hormones. Steroids are ubiquitous in the environment and can be potent endocrine disruptors, even at low concentrations (ng L⁻¹) (Fent, 2015). Steroids with progestogenic activity are called gestagens or progestins. These are synthetic compounds that mimic progesterone activity and are commonly used as oral contraceptives or being part of hormonal replacement therapies. It is proved their adverse effects on fertility and reproduction of aquatic species (Ojoghor et al., 2021), altered sex development, induced transcriptional effects, etc., (Ojoghor et al., 2021; Zhao et al., 2015). Drospirenone (DRO) is a fourth-generation progestin, which was designed to bind the progesterone receptor with greater specificity and minimize side effects related

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to interactions with androgen, estrogen or glucocorticoid receptors, combining potent progestogenic and antiandrogenic activities (Marqueño et al., 2019). Drospirenone can reach the aquatic systems through the effluents of wastewater treatment plants in a range of ng L^{-1} (Fent, 2015; Marqueño et al., 2019). And, there is a lack of knowledge about the effects of this progestin in non-target aquatic species. When it comes to metals, mercury is a high-priority pollutant whose endocrine effects have not been well studied to date (Tan et al., 2009).

According to several authors, human activity manages annually to almost triple the amount of atmospheric mercury (Rice et al., 2014). Once released into the environment, mercury can bioaccumulate and biomagnify in the food web and in the long-term can cause adverse effects on human health (Plunk and Richards, 2020; Tan et al., 2009) and aquatic fauna (Rice et al., 2014). Although the mechanism(s) of mercury entry into the food chain is (are) still unknown, several studies have been developed to understand how it happens (Rice et al., 2014; Tan et al., 2009). About its estrogenic properties, mercury can cause strong negative effects at the reproduction level in both vertebrate and invertebrate species (Tan et al., 2009). Mercury can act at the hypothalamic–pituitary–gonadal (HPG) axis, deregulating its functions at the reproductive level. In the aquatic environment, several studies have demonstrated negative effects of mercury exposure on fish survival, growth rate and external morphology, but also on behavioural characteristics such as hunting, predator avoidance and long-distance migration (Mora-Zamorano et al., 2017; Mora-Zamorano et al., 2016; Webber and Haines, 2003).

Both EDCs (DRO and Hg) can act at interconnecting endocrine axes (as HPG) so they can produce combined effects, impairing the reproductive system and delaying the maturation of the gonads (Fent, 2015; Zucchi et al., 2014). These contaminants associated with climate drivers (e.g., temperature) may produce a stronger and unpredictable effect on the aquatic habitat since the latter can also interfere with the regulation of the HPG axis of vertebrates and invertebrates (Miranda et al., 2013) – Hypothesis 1 (H1).

Regardless of the existent information about the studied stress factors (i.e. temperature, mercury and progestins), individually, less attention has been paid to the environmental health effects of mixtures of EDCs (progestins and metals) under the influence of climate drivers (Cardoso et al., 2017b, 2018a; Mannai et al., 2022). So, it is crucial to evaluate the response of aquatic species to the complex interaction of multiple stressors. Generally, organisms subjected to multiple stressors exhibit one of three types of responses: additive, antagonistic, or synergistic (Todgham and Stillman, 2013), depending also on the timing of occurrence of the stressors. For example, when stressors occur close in time or simultaneously, then interactive effects are more likely to occur (Crain et al., 2008; Gunderson et al., 2016).

Molluscs, in particular gastropods, are ubiquitous in the aquatic system and have been used as non-target models for laboratory studies as they are required to constantly adapt to the many changes that occur in the surrounding environment in which they inhabit (Calabretta and Oviatt, 2008; Cardoso et al., 2017a; Pirger et al., 2018). They proved to be effective model animals because they have highly conserved control and regulatory biochemical pathways that are often homologous to vertebrate systems and they are extremely sensitive to anthropogenic inputs (Pirger et al., 2018). Particularly, *Nucella lapillus* (L.) is a marine gastropod species with strong ecological relevance, occupying the intertidal zone usually associated with rocky shores and common among barnacles (*Balanus* spp.) and mussels (*Mytilus* spp.) on which they preferentially feed (Crothers, 1985; Burrows and Hughes, 1990). It occurs within a salinity range from 18 to 40, and between 0 and 20 °C isotherms, throughout the North Atlantic littoral zone: from the Arctic to the south of Portugal in the east, including Iceland and the Faroe Islands, and from the south-west of Greenland to the north of Long Island in the west (Crothers, 1985; Tyler-Walters, 2007). *Nucella lapillus* is a gonochoristic species (separated sexes) with internal fertilization. Individuals can reproduce throughout the year but, in some places,

reproduction is restricted to a few months (Galante-Oliveira et al., 2010).

The main goal of this study was to evaluate the effects of combined multiple stressors (i.e. temperature and mixtures of the EDCs, Hg and DRO) on the gastropod *Nucella lapillus*. Thus, several endpoints such as survival, fitness (i.e. condition index), consumption rates, and gonads' maturation dynamics were evaluated after 21-day exposure.

2. Materials and methods

2.1. Chemicals

2.1.1. Pharmaceutical

The standard drospirenone (DRO, CAS 67392–87-4; purity = 98.0 %) was purchased from TCI (Tokyo Chemical Industry, Japan). Stock solutions were prepared with analytical ethanol (CAS 64–17-5; purity 99.9 %) supplied by Merck Millipore (Germany) and stored at –20 °C.

2.1.2. Metal

Mercury standard solution ($1000 \text{ mg L}^{-1} \text{ Hg}$) in 10 % nitric acid (for Atomic Absorption Spectrometry) standard was supplied by Fisher Chemical, stored at ambient temperature.

2.2. Organisms' collection and acclimation

The species used in the experiment (i.e. *Nucella lapillus* and its prey, *Mytilus edulis*) were collected in Praia Norte in Viana do Castelo, Portugal ($41^\circ 41'33''\text{N}$ $8^\circ 51'06''\text{N}$) in October 2020 and transported to the CIIMAR facilities in a cool box for acclimation period. The dogwhelk *N. lapillus* individuals were separated by size and adults (total length > 1.5 cm) were selected for the experiment. Also, individuals of *M. edulis* (the most abundant prey species in the field) were selected by size ($2.64 \pm 0.38 \text{ cm}$, total length).

During the acclimation period (15 days) (according to Castro et al., 2007), individuals of *N. lapillus* were fed with *M. edulis* on an ad-libitum basis and maintained in a semi-static system whereby 100 % of the water was changed twice a week. Photoperiod was set to 18 h light: 6 h dark to simulate summer conditions, at constant intensity (1700 lm). The organisms were acclimated under the ambient temperature and normocapnia (18 °C, pH 8.1) and salinity 33–35.

2.3. Experimental design

The experimental set-up followed a factorial design manipulating temperature [ambient temperature (18 °C) (mean sea surface temperature in summer - sSST) (<https://pt.seatemperature.net/current/portugal/viana-do-castelo-viana-do-castelo-portugal>) and warming (22 °C – the future sSST warming scenario in 2100 (+ 4 °C) (Russell et al., 2013)], the progestin drospirenone (DRO: DRO1–100 ng L^{-1} and DRO2–1000 ng L^{-1}), mercury (Hg: Hg1–1.5 $\mu\text{g L}^{-1}$, drinking water's limit, and Hg2–50 $\mu\text{g L}^{-1}$, residual waters' limit), Mixture 1 (Mix 1: Hg1 + DRO1), Mixture 2 (Mix 2: Hg1 + DRO2), Mixture 3 (Mix 3: Hg2 + DRO1), Mixture 4 (Mix 4: Hg2 + DRO2), the Control (Ct: Seawater) and the Solvent control (SCt: Seawater + vehicle ethanol – 0.01 %), in a total of 20 treatments (Table 1), for 21 days (Fig. 1). This experiment followed a similar model to the one already implemented in previous works (Cardoso et al., 2018a) in which a saltwater reservoir tank (500 L) was directly connected to the CIIMAR's internal saltwater network through a 10 μm filter and the flow was maintained by a 1400 L h^{-1} flow pump (Eheim, Germany), that passed first through a UV filter Helix Max 2.0 9 W (Aqua Medic, Germany). Seawater from this reservoir was directed to two tanks (50 L each), whose water flow was later distributed to the 2nd level (i.e. experimental units).

The entire 2nd level was lit by artificial light suitable for marine setups (LED light v-tac, 18w, 240v, 50 Hz, 1700 lm) that was controlled by a timer to guarantee the photoperiod.

Table 1

Description of the different treatments to which *N. lapillus* were exposed. Ct – control, SCt – solvent control, Hg1–1.5 µg L⁻¹, Hg2–50 µg L⁻¹, DRO1–100 ng L⁻¹, DRO2–1000 ng L⁻¹; Mix1–1.5 µg Hg L⁻¹ + 100 ng DRO L⁻¹; Mix2–1.5 µg Hg L⁻¹ + 1000 ng DRO L⁻¹; Mix3–50 µg Hg L⁻¹ + 100 ng DRO L⁻¹; Mix4–50 µg Hg L⁻¹ + 1000 ng DRO L⁻¹.

Treatments	Condition
T1	18 °C, Ct
T2	18 °C, SCt
T3	18 °C, Hg1
T4	18 °C, Hg2
T5	18 °C, DRO1
T6	18 °C, DRO2
T7	18 °C, Mix1
T8	18 °C, Mix2
T9	18 °C, Mix3
T10	18 °C, Mix4
T11	22 °C, Ct
T12	22 °C, SCt
T13	22 °C, Hg1
T14	22 °C, Hg2
T15	22 °C, DRO1
T16	22 °C, DRO2
T17	22 °C, Mix1
T18	22 °C, Mix2
T19	22 °C, Mix3
T20	22 °C, Mix4

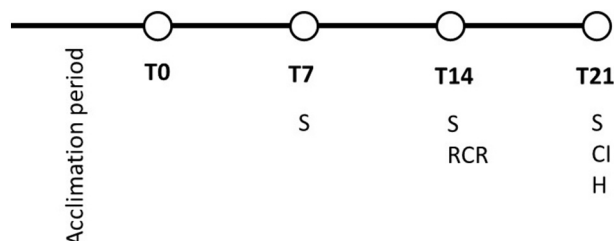


Fig. 1. Graphical representation of the timeline of the experiment with indication of the endpoints analysed at each sampling point. S – survival, RCR – relative consumption rate, CI – condition index and H – Histological analysis. The acclimation period corresponds to the 15 days acclimation to the lab conditions plus 4 days of warming increment till the 22 °C condition.

In all 2nd level flasks, aeration and the saltwater flow were maintained continuously (0.6 L h⁻¹ flask⁻¹). Finally, every 4 flasks were linked by connecting vessels to another flask, external to the water baths, so that all the contaminated water passed through a set of particle filters with 3 different meshes (5, 10 and 25 µm), then a charcoal filter, before being eliminated.

Specimens were divided by treatments and each one was composed of four replicates in a total of eighty glass flasks (2 L-volume, 9.7 cm in diameter). Replicates were distributed randomly by ten water baths, to maintain the temperature constant (Fig. 2). The temperature inside the water baths was maintained by 300 KW h⁻¹ resistors that were regulated by a temperature sensor controlled by AT Control power box (Aqua Medic, Germany), which automatically heated the tanks whenever the temperature deviated from predetermined set points by 0.5 °C. Each flask had twenty-one *N. lapillus* (eighteen were maintained free in the flasks plus three in isolated perforated plastic flasks for control of consumption rate) that were maintained at salinity 33–35, and a photoperiod of 16 h light: 8 h dark (summer conditions), with a constant water renovation and aeration. During the experiment, the organisms in the flasks were directly exposed to Hg and DRO three times a day (200 µL of each stock solution for each contaminant concentration was injected directly into the flasks), to simulate episodic discharges from a contamination source.

Following previous works (Cardoso et al., 2018a; Potts et al., 2021), the water temperature was increased gradually (1 °C per day) until reaching the highest temperature of 22 °C. After reaching the required temperature the experiment started. During the 21 days of exposure, individuals were fed with mussels (one mussel per three dog-whelks) replaced three times a week (corresponds approximately to 1 mussel/dog-whelk/week). Attending to the literature, the feeding rate can be very variable depending on the size of prey, temperature (Crothers, 1985). So, we considered an average value based on the literature (Crothers, 1985 and references therein: 0.28–0.77 mussels/week and 3.5 mussels/week; Hunt and Sheibling, 1998: 0.7–1.09 mussels/week).

Survival was checked on days 7, 14 and 21 ($n = 21$ individuals/replicate).

During the experiment, water physicochemical variables, such as temperature, pH, dissolved oxygen, ammonia and nitrites were measured in the experimental units three times a week, and the temperature was measured daily. During the experiment the seawater environmental variables were maintained as follows: temperature: 18.3 ± 0.11 °C and 21.87 ± 0.23 °C; pH: 7.92 ± 0.07 (18 °C) / 9.81 ± 0.06 (22 °C); dissolved oxygen: 98.9 ± 0.45 % (18 °C) / 97.6 ± 4 % (22 °C); ammonia: 0.11 ± 0.03 mg L⁻¹ (18 °C) / 0.14 ± 0.04 mg L⁻¹ (22 °C) and nitrites: 0.19 ± 0.04 mg L⁻¹ / 0.21 ± 0.06 mg L⁻¹ (22 °C) (please check Tables S1 and S2 for more details). The experiment was carried out at CIIMAR aquatic animal facilities, according to the guidelines of the Directorate-General of Veterinary of Portugal (Decree-Law No. 113/2013), implementing the European Directive No. 2010/63/EU on animal welfare for scientific purposes.

2.4. Hg quantification in the water

Water samples (150 mL, $n = 2$ replicates per treatment) were collected from all exposure groups just after the Hg addition (T30–30 min after and T60–60 min after) and preserved in glass flasks containing 0.7 mL HCl 21 %. Total dissolved mercury was quantified through ICP by Biogerm, S.A. (Portugal).

2.5. Drosipreneone (DRO) quantification in water samples by LC-MS

2.5.1. Water sampling

Water samples (1 L, $n = 2$ replicates per treatment) from all exposure groups were collected in amber flasks just after DRO addition (T30–30 min after and T60–60 min after). During sampling, all bottles were rinsed twice with the treated water before collection. Then, water samples were filtered with GF/C™ glass microfiber filters from Whatman (1.2 µm; 47 mm; Lot No.:16836949) and stored at –20 °C until analysis.

2.5.2. Extraction procedure

The extraction method followed (Ribeiro et al., 2007), initially developed to extract phenolic compounds and steroids from water samples, with some modifications. The filtered samples were treated (cleaned and concentrated) by solid-phase extraction using SPE cartridges from Waters™ Corporation (Milford, MA, USA; Oasis® HLB 6 cc/200 mg). Prior to use, cartridges were sequentially conditioned with 12 mL of acetonitrile:ethyl acetate (50:50, v/v) and 12 mL of ultrapure Milli-Q water (VWR, EUA). Samples of 1 L were, then, loaded onto SPE cartridges, at a constant flow rate of approximately 5 mL min⁻¹ followed by a washing step with 6 mL of ultrapure Milli-Q water. Cartridges were dried under vacuum for 15 min and, then, eluted with 6 mL of acetonitrile:ethyl acetate (50:50, v/v), at 1 mL min⁻¹. Elution volume was completely evaporated under a nitrogen stream and reconstituted in 1 mL methanol (LC-MS grade) acidified with formic acid (0.1 %), concentrating the original samples 1000 times.

2.5.3. Instrumental and methodological characteristics

Samples were injected (2×20 µL) into a Liquid Chromatograph

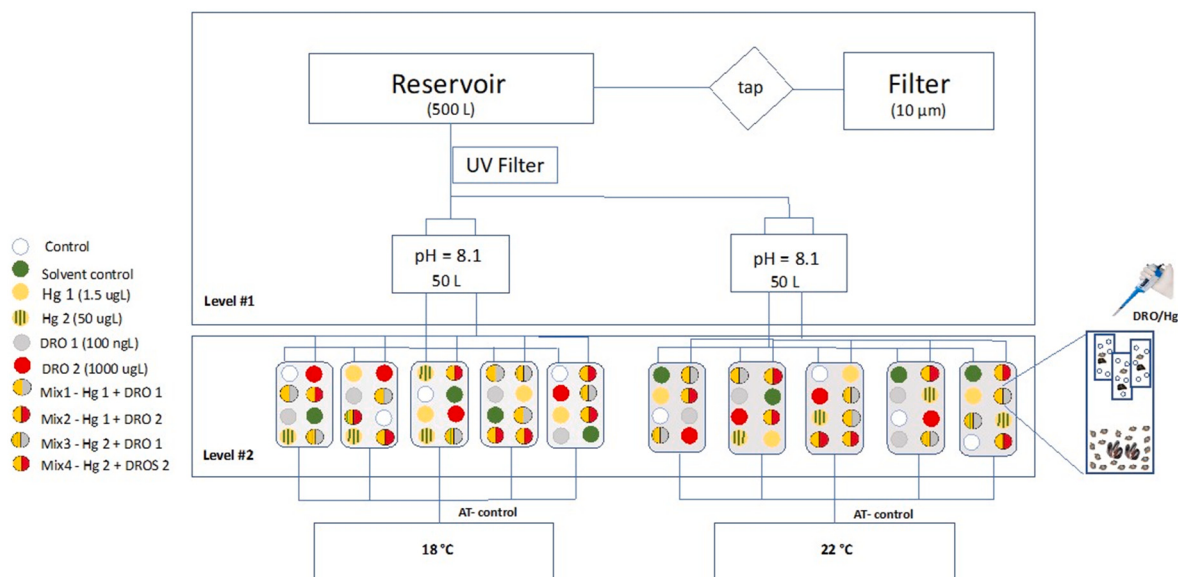


Fig. 2. Schematic representation of the experimental set-up of the mesocosm experiment with *N. lapillus*. Level #1 represents the saltwater reservoir tank (500 L) directly connected to the internal saltwater network through a 10 µm filter and the flow is distributed to two main tanks that feed each temperature system (one for 18 °C and the other for 22 °C) of experimental units (2nd level). Level #2 represents the 10 water baths, for the 2 temperatures. Each water bath has 8 experimental units (i.e. flasks) corresponding to different treatments distributed randomly. In total, for each temperature there are 40 experimental units (10 treatments × 4 replicates). Drosiprenone and mercury are injected directly in the flasks.

Thermo Finnigan Surveyor HPLC System (Thermo Scientific, MA, USA), coupled to an ESI source-Mass Spectrometry LCQ Fleet™ Ion Trap Mass Spectrometer (Thermo Scientific, MA, USA). Separation was achieved with a column Avantor® ACE Excel (50 mm × 2.1 mm i.d., 1.7 µm) (Avantor-LotV19-3430; Serial No.: A210214175) kept at 25 °C and at a flow rate of 0.15 mL min⁻¹.

The mass spectrometer was operated in positive mode at: spray voltage of 4.55 V; capillary voltage of 30 V; capillary temperature of 380 °C; tube lens of 55 V and normalised collision energy maintained at 35. Data were processed using Xcalibur™ version 2 Software.

A summary of analysis detection parameters for Drosiprenone can be

weight (SW) was calculated as FW-TW. The condition index was estimated as (TW/SW) × 100, according to (Mamo et al., 2019) at the end of the experiment.

Relative consumption rates (RCRs) were evaluated during the second week of the experiment ($n = 3$ individuals/replicate). For that, were used three individualized *N. lapillus* (plus three *M. edulis*; one per dogwhelk) per replicate. The individuals selected for this assessment were starved for 24 h before the consumption experiment that run for 3 days.

N. lapillus consumption rate (RCR) was determined according to (Guler and Lök, 2019) and calculated as mussel (g). snail (g)⁻¹. day⁻¹ as follows:

$$RCR = \left(\frac{\text{initial average wet weight mussel (g)} - \text{final wet weight mussel (g)}}{\text{average wet weight snail (g)}} \right) / 3 \text{ days}$$

seen in Table S3 (supplementary material).

After integration, samples were mathematically quantified by an external standard-matrix calibration curve (method of least squares) with eight nominal concentrations from 10 to 2000 µg L⁻¹ (which is equivalent to 10 to 2000 ng L⁻¹).

Recovery tests were performed using sample-matrix doped with a three-level concentration, to have 500, 1000 and 2000 µg L⁻¹ concentrations in LC-MS analysis. The recovery of the method was 69 ± 6 %. The limit of detection (LOD) is 10 µg L⁻¹ and the limit of quantification (LOQ) is 20 µg L⁻¹. Nominal values below 20 µg L⁻¹ and 10 µg L⁻¹ were indicated as <LOQ and < LOD, respectively and no detected values as ND.

2.6. *N. lapillus* condition index and consumption rate

For estimation of the *N. lapillus* condition ($n = 4$ individuals/replicate), the fresh weight (FW, i.e. body weight plus shell weight) and the wet tissue excluding the operculum (TW) were measured using an analytical precision scale (Acculab sartorius group, Germany) and shell

2.7. Histological procedures

Organisms selected for histological analysis ($n = 3$ individuals/replicate) were fixed individually in bouin solution (ITW Reagents, Spain) in small plastic flasks, for 48 h. Afterwards, they were transferred to ethanol 70 %. After 24-48 h in ethanol, this was replaced with a clean one.

After fixation, tissues were processed in an automatic processor (Citadel 2000, Thermo Scientific, USA). Tissues were embedded in paraffin (Histoplast IM, Thermo Scientific, USA) and left to cool. Embedded tissues were cut into 6 µm longitudinal semithin sections in a paraffin microtome (Jung RM 2035, Leica Biosystems, Germany). Finally, sections were stained in an automatic slide stainer (Shandon Varistain 24-4, Thermo Scientific, USA) with haematoxylin-eosin and mounted in Entellan new resin (Merck Millipore, Germany) for light microscopy observation to determine the individual gametogenic stage.

2.8. Gonads' microscopic evaluation

For the microscopic examination of gonads' maturation, it was used a classification based on the previous work of Galante-Oliveira et al. (2010), in which a 6-stage scale was established: I (immature), II (early recovering), III (late recovering), IV (ripe), V (partially spent) and VI (spent).

Attending to the variability in the maturity of the gonads it was decided to analyse 5 follicles from each gonad section, in a total of 45 follicles (5 follicles $\times$ 3 slides $\times$ 3 sections) and the individual gametogenesis stage considered was the median value among those registered for the 45 observed follicles (following the same procedure as in Cardoso et al., 2018b).

2.9. Data analysis

Experimental results were examined using linear models. All the statistical analyses were run in R environment (Team, 2016). Initially, models for all the responses were constructed including fixed factors and random effects (i.e. generalized linear mixed models, LMM). However, when random effects did not improve the model fit, we applied the parsimonious principle, removed those terms and used generalized linear models provided by glm2 package in R (Marschner, 2018). Hence, to examine the effects of the treatments on the survival of *N. lapillus* at the end of the experiment, we used GLM assuming a Binomial data distribution, as there were only two possible outcomes (alive and dead). To visualize the survival curves for all the treatments, which are represented as survival percentages, we used the function ggsurvplot provided by Survminer R package (Kassambara et al., 2021). The model included as predictors the three experimental factors: temperature level, Hg and DRO concentrations, but also their interactions. Analogous linear models were constructed to examine consumption rates, but this time data using a Gaussian data distribution. In the case of the analysis of gonads' maturation we used an ordered logist regression model, according to Galante-Oliveira et al. (2010), because the response was an ordered categorical outcome (6-stage condition scale). Model was constructed in the same way as described above.

In all the models, significant predictors were selected from the full models by removing sequentially those of higher-order, with the higher *p* values, and comparing the reduced model with the original one using analysis of variance (ANOVA). When significant interactions were

found, treatments were compared using *p*-adjusted Tukey tests (*p* < 0.05). The Lsmmeans package for R was used to perform these tests a posteriori (Lenth, 2016).

Generalized linear mixed models ran using nlme package (Pinheiro et al., 2012). Ordered logit linear models were constructed using the package MASS (Venables & Ripley, 2002). Assumptions for the linear models were checked by examining the residual plots. In the case of response variables with Gaussian probability distribution, and, when required, data were log-transformed to avoid heteroscedasticity.

3. Results

3.1. Mercury in water

Mercury concentrations measured at T30 (30 min) and T60 (60 min) after the first injection are indicated in Table 2. Measured concentrations in control (Ct) and solvent control (SCt) were close to zero. At T30, concentrations declined around 60 % for the lowest concentration (Hg1) and approximately 80 % for the highest concentration (Hg2). At T60 they decreased even more ($\approx$ 40–50 % in relation to T30) (Table 2). For the mixtures, the losses were more variable.

3.2. Drospirenone in water

Drospirenone concentrations measured at T30 (30 min) and T60 (60 min) after the first injection are indicated in Table 3. In all control (Ct) and solvent control (SCt) samples, DRO was not detected. For the rest of the treatments, values at T30 were lower than the nominal values, except for DRO 2 (T18 °C) and Mix 1 (T22°C) which presented values slightly higher than nominal concentrations. At T60, the values declined considerably (Table 3).

3.3. Survival

For all the endpoints analysed, was considered only the solvent control (SCt) against the other treatments, since no significant differences between control and solvent control were observed.

The survival rate was estimated along 21 days, with checking points on days 7, 14 and 21.

For the SCt group, at both temperatures, no lethality was observed

Table 2

Nominal and measured concentrations of mercury ($\mu\text{g L}^{-1}$) in waters collected during the experiment. The water was sampled 30 min after the first injection (T30) and 60 min later (T60). The values are expressed as the mean ($\pm$ SD). Ct – control, SCt: solvent control – 0.01 % ethanol; Hg1–1.5 $\mu\text{g L}^{-1}$, Hg2–50 $\mu\text{g L}^{-1}$; Mix1–1.5 $\mu\text{g Hg L}^{-1}$ + 100 ng DRO L^{-1} ; Mix2–1.5 $\mu\text{g Hg L}^{-1}$ + 1000 ng DRO L^{-1} ; Mix3–50 $\mu\text{g Hg L}^{-1}$ + 100 ng DRO L^{-1} ; Mix4–50 $\mu\text{g Hg L}^{-1}$ + 1000 ng DRO L^{-1} .

Treatments	Nominal ($\mu\text{g L}^{-1}$)	T30 ($\mu\text{g L}^{-1}$)	T60 ($\mu\text{g L}^{-1}$)
18 °C			
Ct	–	< 0.01	0.039 $\pm$ 0.025
SCt	–	0.057 $\pm$ 0.028	< 0.01
Hg1	1.5	0.487 $\pm$ 0.057	0.342 $\pm$ 0.095
Hg2	50	8.475 $\pm$ 1.28	3.28 $\pm$ 0.82
Mix1	1.5	–	–
Mix2	1.5	0.231 $\pm$ 0.094	0.105 $\pm$ 0.05
Mix3	50	0.485 $\pm$ 0.12	0.342 $\pm$ 0.205
Mix4	50	4.29	0.86
22 °C			
Ct	–	0.017	0.020
SCt	–	< 0.01	0.042 $\pm$ 0.034
Hg1	1.5	0.75 $\pm$ 1.047	0.342 $\pm$ 0.095
Hg2	50	7.76 $\pm$ 1.87	3.91 $\pm$ 1.527
Mix1	1.5	0.16	0.114
Mix2	1.5	0.527 $\pm$ 0.087	0.398 $\pm$ 0.035
Mix3	50	14.2 $\pm$ 3.394	0.642 $\pm$ 0.06
Mix4	50	16.3 $\pm$ 4.243	6.68 $\pm$ 1.047

Table 3

Nominal and measured concentrations of drospirenone (ng L^{-1}) in waters collected during the experiment. The water was sampled 30 min after the first injection (T30) and 60 min later (T60). The values are expressed as the mean ($\pm$ SD). Ct – control, SCt: solvent control – 0.01 % ethanol; DRO1–100 ng L^{-1} , DRO2–1000 ng L^{-1} ; Mix1–1.5 $\mu\text{g Hg L}^{-1}$ + 100 ng DRO L^{-1} ; Mix2–1.5 $\mu\text{g Hg L}^{-1}$ + 1000 ng DRO L^{-1} ; Mix3–50 $\mu\text{g Hg L}^{-1}$ + 100 ng DRO L^{-1} ; Mix4–50 $\mu\text{g Hg L}^{-1}$ + 1000 ng DRO L^{-1} . ND – not detected.

Treatments	Nominal (ng L^{-1})	T30 (ng L^{-1})	T60 (ng L^{-1})
18 °C			
Ct	–	ND	ND
SCt	–	ND	ND
DRO1	100	86.31 $\pm$ 10.47	< LOD
DRO2	1000	1249.16 $\pm$ 665.64	757.69 $\pm$ 35.21
Mix1	100	129.15	< LOD
Mix2	1000	–	–
Mix3	100	61.21 $\pm$ 12.5	< LOQ
Mix4	1000	965.06 $\pm$ 56.88	583.08 $\pm$ 92.54
22 °C			
Ct	–	ND	ND
SCt	–	ND	ND
DRO1	100	54.41	<LOD
DRO2	1000	583.01 $\pm$ 402.42	339.73
Mix1	100	298.98	33.32 $\pm$ 29.4
Mix2	1000	892.52	244.04 $\pm$ 58.49
Mix3	100	107.34 $\pm$ 1.10	< LOQ
Mix4	1000	749.06	59.75 $\pm$ 46.62

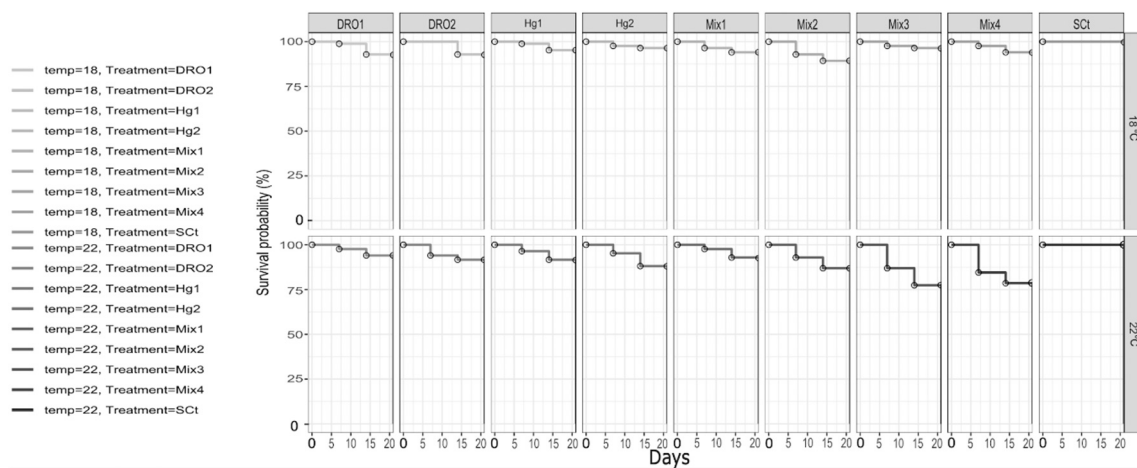


Fig. 3. Survival (%) of *N. lapillus* exposed to different combinations of temperature (18 and 22 °C), Hg (Hg1–1.5 µg L⁻¹, Hg2–50 µg L⁻¹) and DRO concentrations (SCt – solvent control, DRO1–100 ng L⁻¹, DRO2–1000 ng L⁻¹; Mix1–1.5 µg Hg L⁻¹ + 100 ng DRO L⁻¹; Mix2–1.5 µg Hg L⁻¹ + 1000 ng DRO L⁻¹; Mix3–50 µg Hg L⁻¹ + 100 ng DRO L⁻¹; Mix4–50 µg Hg L⁻¹ + 1000 ng DRO L⁻¹). (n = 21 per treatment).

after 21 days of experiment. On the other hand, DRO and/or Hg treatments suffered a negative impact on survival rate. But, the warming condition associated with the presence of Hg and/or DRO had a greater

negative impact on the survival of *N. lapillus*, since the treatments exposed to 22 °C were particularly affected on day 21, reaching 65–80 % survival rate compared with almost 90 % of survival for those exposed to

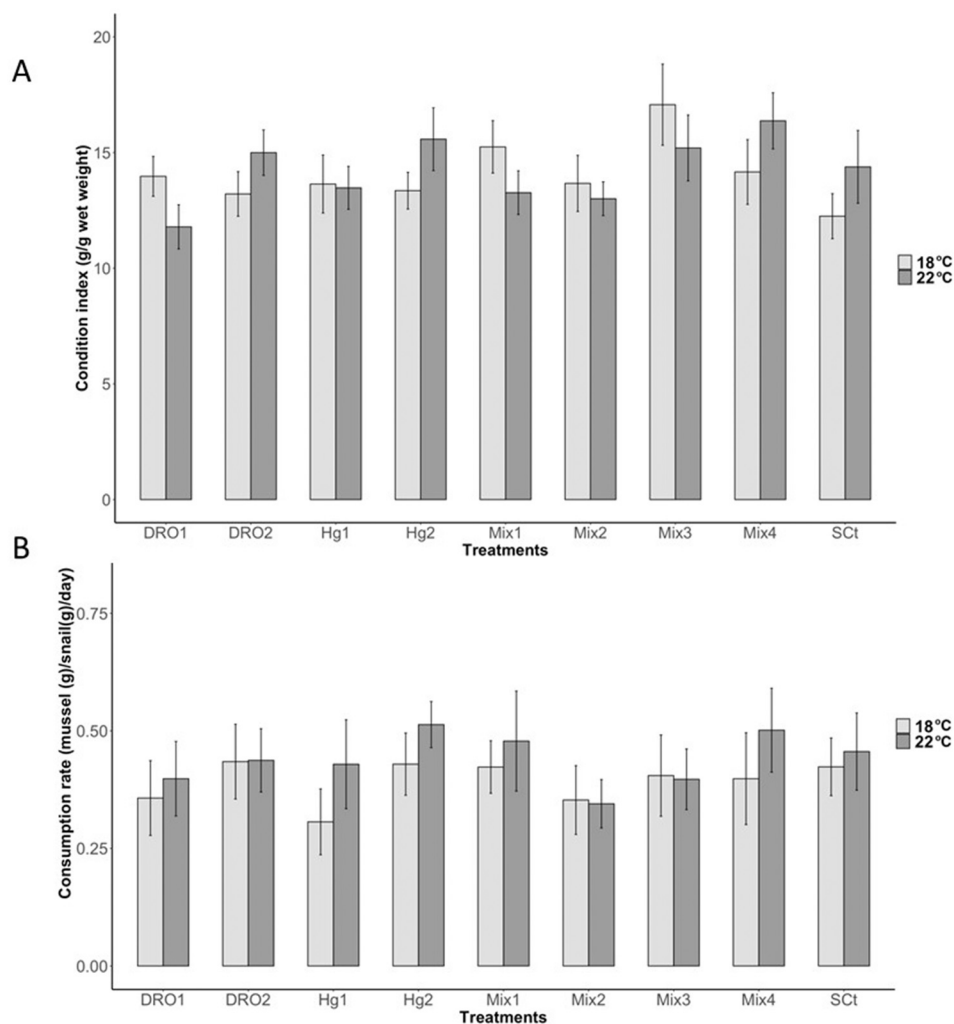


Fig. 4. Condition index (A) and consumption rates (B) of *N. lapillus* exposed to the distinct treatments (n = 12 per treatment) for the trial period (21 days). SCt: solvent control – 0.01 % ethanol; Hg1–1.5 µg L⁻¹, Hg2–50 µg L⁻¹; DRO1–100 ng L⁻¹, DRO2–1000 ng L⁻¹; Mix1–1.5 µg Hg L⁻¹ + 100 ng DRO L⁻¹; Mix2–1.5 µg Hg L⁻¹ + 1000 ng DRO L⁻¹; Mix3–50 µg Hg L⁻¹ + 100 ng DRO L⁻¹; Mix4–50 µg Hg L⁻¹ + 1000 ng DRO L⁻¹. Values represent mean (± SD).

18 °C. Mix 2, 3 and 4 were the ones that presented the lowest survival rates (74–79 %), except DRO1 (65 %), (Fig. 3).

On day 7, the survival rates were quite similar for all the treatments (97–100 %), except for Mix 2, 3 and 4 which were lower (84–92 %). On day 7, significant effects of temperature, Hg and DRO, as well as a significant interaction between temp:Hg, were observed (GLM model, $p < 0.05$), (Table S4).

On day 14, significant effects of temp, Hg and DRO, as well as on the interaction between temp:Hg:DRO, were observed (GLM model, $p < 0.05$), (Table S5). On day 21, a significant effect of temp, Hg and DRO as isolated factors, as well as interactions between temp:DRO, and between Hg:DRO, were observed (Table S6).

3.4. Condition index and consumption rate

The condition index, estimated at the end of the experimental period (i.e., after the 21 days), for both temperatures, revealed no statistically significant differences among all the treatments (LMM model, $p > 0.05$), (Fig. 4A).

According to the results obtained, consumption rates were very homogeneous throughout the treatments, for both temperatures, with no significant differences among them (GLS model, $p > 0.05$), (Fig. 4B).

3.5. Histological analysis of gonads' maturation

According to Fig. 5, at the ambient temperature (T1), the general maturation stage in females was III-IV while in males was IV-V. In the meanwhile, in a scenario of warming (T2), we could observe a general delay in the maturation stage of the ovaries, being observed a tendency for the occurrence of follicles in stage III to the detriment of IV. In the case of males, this pattern was not visible.

Analysing the percentage of occurrence of the distinct maturation stages in the ovary of *N. lapillus*, in the solvent control (SCt) condition at 18 °C, 80 % of the females were classified as being in stage IV and 20 % in stage V. However, when they were exposed to the different chemicals (Hg and/or DRO) in combination with the increase of temperature (T2), the percentage of non-mature follicles increased (Fig. 6), except for Mix1 (all females in stage IV). In males, a similar pattern was observed: in SCt most of the individuals were in stages V and VI while, in the presence of contaminants and/or elevated temperature, less mature stages occurred.

In Fig. 7 are represented different stages of gametogenesis in which is visible a decline in the maturation stages, of both males and females, from SCt to other treatments (EDCs and/or warming), which confirms the pattern observed in Fig. 5. For example, females of SCt were mainly in stage IV, but when exposed to mixtures of EDCs at higher

temperature, other less mature stages I, and the frequency of occurrence of stage IV decreased. In males, a similar pattern was observed: in SCt males were in stages V and VI while, when exposed to EDCs, other less mature stages appeared (e.g. II, III and IV).

The results of the ordered logit regression model are shown in Table 4 and reinforce the previous results indicating that the males are in a more advanced gametogenic stage than females (Table 4, line 18). However, the highest temperature in combination with both concentrations of mercury (Hg1 and Hg2), (Table 4, rows 5–6), and mixtures of both contaminants (Table 4, row 9–10), had an inhibitory effect on female gonads' maturation.

In males, we found that the effect was also inhibitory at the lowest temperature (18 °C) and in the presence of low Hg concentrations (Hg1), (Table 4, line 19). A similar effect was observed again at 22 °C for Mix 1 (Hg1 + DRO1), (Table 3 row 30), Mix 3 (Hg2 + DRO1) and Mix 4 (Hg2 + DRO2), (Table 3 rows 32–33).

The lower part of Table 4 (lines 34–37) specifies the thresholds (also referred to as cut-off points) that indicate the correspondence between a continuous variable and the different gametogenic maturation stages of the model species and the stages that were observed at the microscopic level. It should be noted that there was a negative effect of the compounds used concerning the development of the gonads, especially in the earlier stages of maturation (Table 4, rows 34–35) ($p < 0.001$).

4. Discussion

In the last decades, many studies have focused on the effects of progestins, first isolated and, more recently, as mixtures of steroid hormones, demonstrating the negative effects of these compounds on aquatic species (Liang et al., 2019, Schmid et al., 2020, Fent, 2015). However, to our knowledge, there is a lack of information about the combined effects of climate variables (e.g. temperature) and mixtures of EDCs, such as a progestin and a metal, namely mercury. So, this work breakthrough the state of the art, contributing with novel information regarding these hot topics.

According to our findings, the exposure of *Nucella lapillus* to DRO or Hg caused negative effects on the survival of the gastropod, but those effects were much more exacerbated in the presence of mixtures of both contaminants and at a higher temperature. This negative effect of warming on survival was similar to the observed by Falkenberg et al. (2021) for the gastropod species *Chlorostoma argyrostoma* and *Lunella granulate* in which was observed a decline of 10–20 % in the survival rate when exposed to increased temperature (+ 2.5 °C) after 40 days of exposure. A similar result was observed for the crustacean *Gammarus locusta* (Cardoso et al., 2018a). However, in the present work, there was

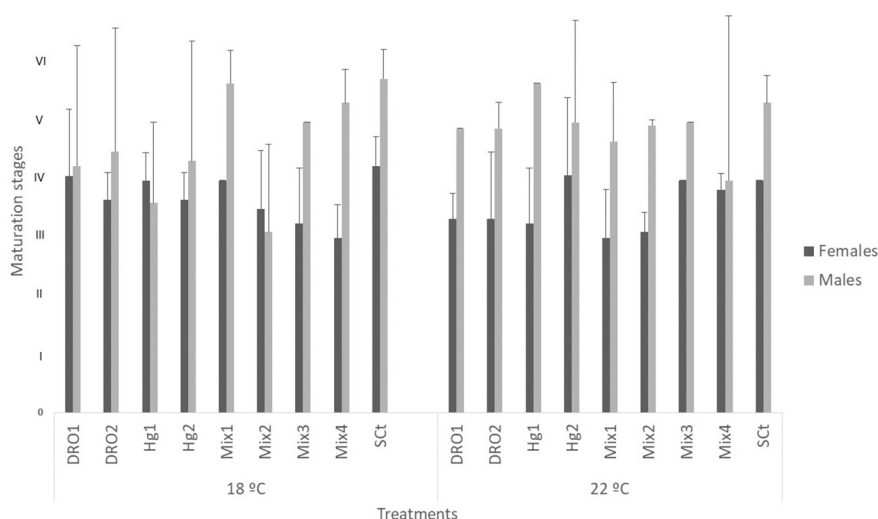


Fig. 5. Maturation stages – I (immature), II (early recovering), III (late recovering), IV (ripe), V (partially spent) and VI (spent) – of the gastropod *N. lapillus* ovary and testis exposed to the different treatments. SCt: solvent control – 0.01 % ethanol; Hg1–1.5 µg L⁻¹, Hg2–50 µg L⁻¹; DRO1–100 ng L⁻¹, DRO2–1000 ng L⁻¹; Mix1–1.5 µg Hg L⁻¹ + 100 ng DRO L⁻¹; Mix2–1.5 µg Hg L⁻¹ + 1000 ng DRO L⁻¹; Mix3–50 µg Hg L⁻¹ + 100 ng DRO L⁻¹; Mix4–50 µg Hg L⁻¹ + 1000 ng DRO L⁻¹. Data are represented as median (± SD).

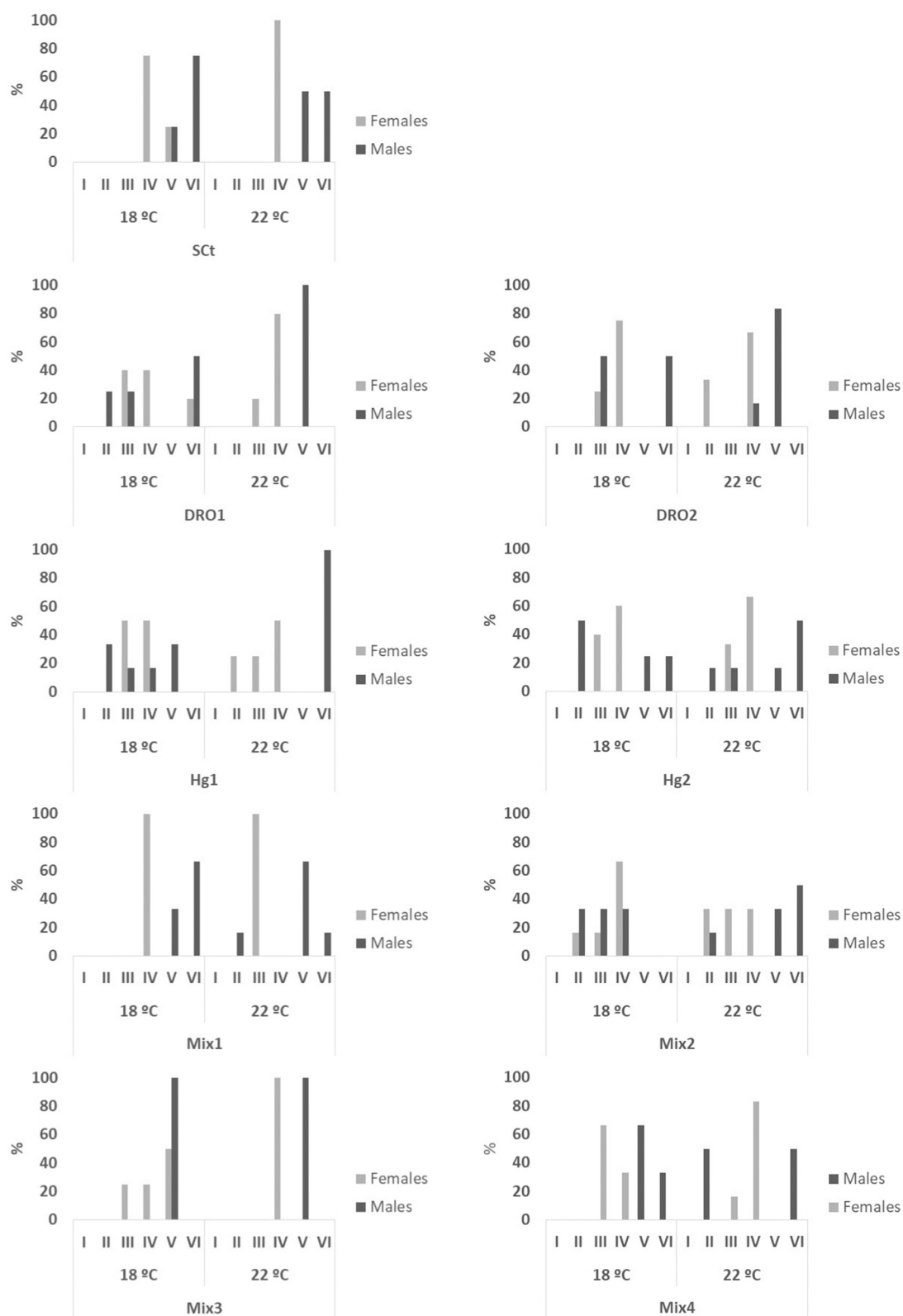


Fig. 6. Percentage of occurrence of the maturation stages of females and males of *N. lapillus* exposed to the different treatments. SCt: solvent control – 0.01 % ethanol; Hg1–1.5 $\mu\text{g L}^{-1}$, Hg2–50 $\mu\text{g L}^{-1}$; DRO1–100 ng L^{-1} , DRO2–1000 ng L^{-1} ; Mix1–1.5 $\mu\text{g Hg L}^{-1}$ + 100 ng DRO L^{-1} ; Mix2–1.5 $\mu\text{g Hg L}^{-1}$ + 1000 ng DRO L^{-1} ; Mix3–50 $\mu\text{g Hg L}^{-1}$ + 100 ng DRO L^{-1} ; Mix4–50 $\mu\text{g Hg L}^{-1}$ + 1000 ng DRO L^{-1} .

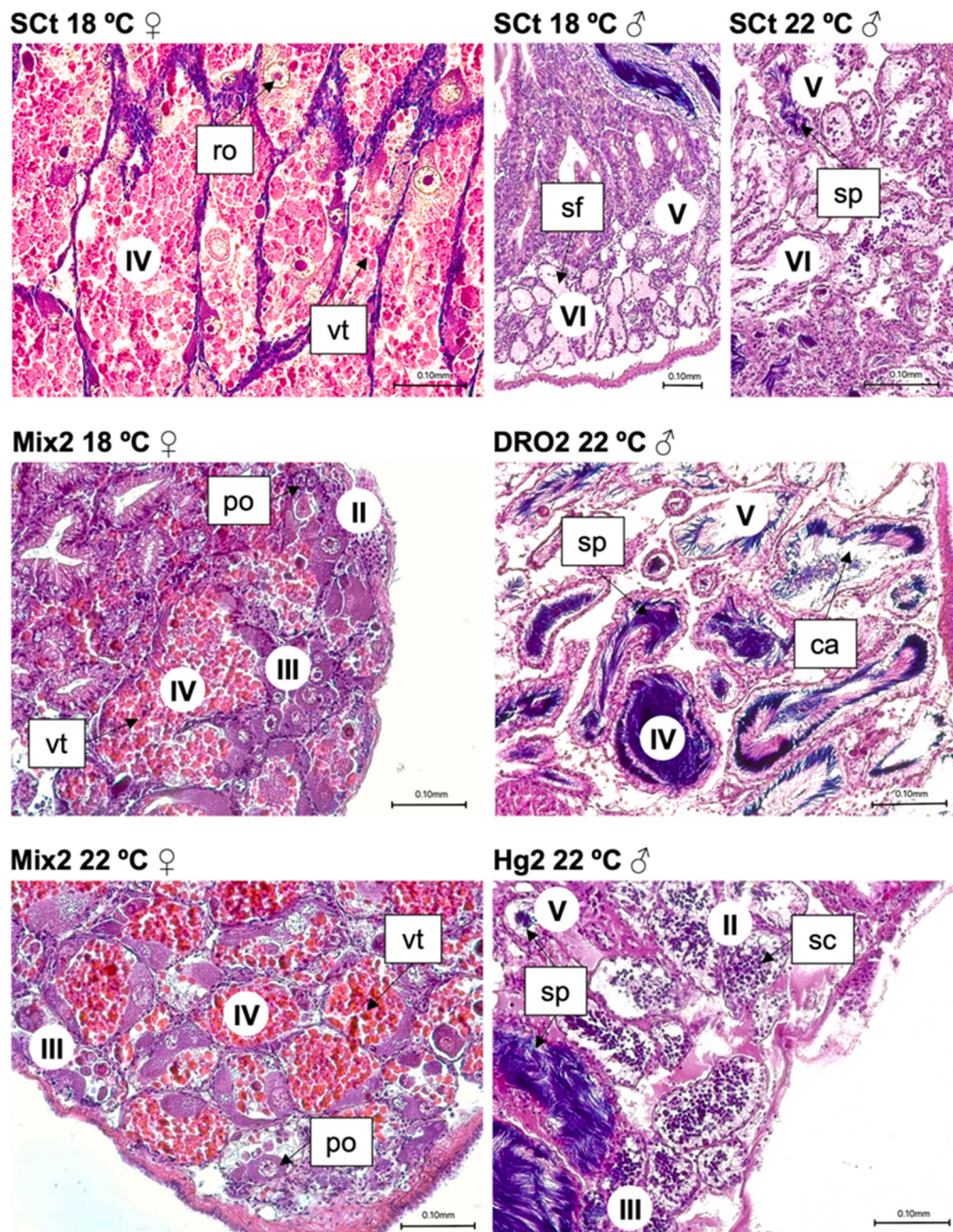


Fig. 7. Gametogenic stages (II to VI) identified in *N. lapillus* ovaries (left column) and testis (right column) after exposure to the experimental treatment indicated on each picture top-left corner. Ca: cavity after sperm shed; po: previtellogenic oocyte; ro: ripe oocyte; sc: spermatocyte; sf: spent follicle; sp.: spermatozoa; vt: vitellum.

a clear effect of DRO and/or Hg, even at low concentrations and ambient temperature, in the survival of *N. lapillus*. An opposite result was observed for *Daphnia magna* when exposed to a mixture of progestogens (Svighu et al., 2021): no lethality was observed at any concentrations during 21 days of exposure.

Regarding possible effects of the studied stressors on the condition of *N. lapillus* and consumption rates, there were no significant impacts. Similar results were observed by Falkenberg et al. (2021) regarding effects of increased temperature on feeding and oxygen consumption rates of gastropods *C. argyrostoma* and *L. granulate*. However, in a previous study by Cardoso et al. (2018a), it was observed a significant positive effect of levonorgestrel (lowest concentration) on the consumption rates of the crustacean *Gammarus locusta*. Also, it was observed a significant negative effect of increased temperature on the condition of *G. locusta*.

Concerning the effects of studied EDCs on the gonads' maturation

dynamics, it was clear that DRO and Hg as isolated factors did not affect *N. lapillus*, even at the highest concentrations tested. A similar effect of drospirenone was observed by Cappello et al. (2017) in the bivalve *Mytilus galloprovincialis*, when exposed to concentrations ranging from 20 to 10,000 ng L⁻¹, for 7 days. However, in our study, we could observe that DRO and Hg in association with increased temperature had a synergistic effect, delaying the maturation stage of the gonads, particularly in females. So, once again, the temperature increase has a crucial impact on the vital functions of the organisms, exacerbating the effects of isolated factors, and affecting the reproductive system as expected (H1). Additionally, it was observed in both sexes an increase in the frequency of earlier maturation stages with the exposure to DRO and/or Hg under warming. In another study by Zeilinger et al. (2009), they observed a decline in the percentage of mature vitellogenic stage oocytes of fathead minnow, from 26 to 2 % after 21 days of exposure to 70 µg L⁻¹ of

Table 4

Results of the ordered logit model for the ordinal variable “maturation stage”. SCt: solvent control – 0.01 % ethanol; Hg1–1.5 $\mu\text{g L}^{-1}$, Hg2–50 $\mu\text{g L}^{-1}$; DRO1–100 ng L^{-1} , DRO2–1000 ng L^{-1} ; Mix1–1.5 $\mu\text{g Hg L}^{-1}$ + 100 ng DRO L^{-1} ; Mix2–1.5 $\mu\text{g Hg L}^{-1}$ + 1000 ng DRO L^{-1} ; Mix3–50 $\mu\text{g Hg L}^{-1}$ + 100 ng DRO L^{-1} ; Mix4–50 $\mu\text{g Hg L}^{-1}$ + 1000 ng DRO L^{-1} .

	Line	Coefficients	Value	t-Value	p- Value
♀	1	Sex: Mix1	1.1675	1.1628	2.45E-01
	2	Sex: Mix2	0.2465	0.2977	7.66E-01
	3	Sex: Mix3	0.3254	0.3261	0.7444
	4	Sex: Mix4	0.3814	0.4509	6.52E-01
	5	Sex: temp22: Hg1	−3.6189	−2.5879	9.66E-03 **
	6	Sex: temp22: Hg2	−3.3652	−2.5835	9.78E-03 **
	7	Sex: temp22: DROS1	0.1225	0.1470	0.8831
	8	Sex: temp22: DROS2	−0.0586	−0.0623	0.9503
	9	Sex: temp22: Mix1	−0.8569	−0.8155	0.4148
	10	Sex: temp22: Mix2	−0.2165	−0.2532	0.8001
	11	Sex: temp22: Mix3	2.0826	2.0779	0.0377 *
	12	Sex: temp22: Mix4	1.4277	1.6910	0.0908
♀ vs ♂	13	Temp22	2.9260	2.6634	7.74E-03 **
	14	Hg1	−1.0183	−1.2873	1.98E-01
	15	Hg2	−1.0182	−1.7496	8.02E-02
	16	DROS1	−0.4709	−0.7414	4.58E-01
	17	DROS2	−1.0183	−1.6380	1.01E-01
	18	Sex	2.0530	3.7915	1.50E-04 *
	19	Sex: Hg1	−2.1038	−2.0251	4.29E-02 *
♂	20	Sex: Hg2	−1.6872	−1.4519	1.47E-01
	21	Sex: DROS1	−0.9304	−0.7246	4.69E-01
	22	Sex: DROS2	−0.1252	−0.0827	9.34E-01
	23	Sex: temp22: Hg1	1.9881	1.1610	2.46E-01
	24	Sex: Mix1	6.3153	4.6382	3.52E-06 **
	25	Sex: Mix2	−0.1361	−0.0889	9.29E-01
	26	Sex: Mix3	4.2097	2.7155	6.62E-03 **
	27	Sex: Mix4	4.5639	2.6403	8.28E-03 **
	28	Sex: temp22: DROS1	0.5406	0.3549	0.7226
	29	Sex: temp22: DROS2	0.0542	0.0360	0.9713
	30	Sex: temp22: Mix1	−6.9056	−4.7225	< 0.001 ***
	31	Sex: temp22: Mix2	1.9438	1.5716	1.16E-01
	32	Sex: temp22: Mix3	−4.9141	−3.4157	< 0.001 ***
	33	Sex: temp22: Mix4	−5.5307	−3.1325	< 0.001 ***
	34	2 3	−2.7482	−6.7799	< 0.001 ***
	35	3 4	−1.5633	−4.0091	< 0.001 ***
	36	4 5	0.9199	2.4053	0.0162 **
	37	5 6	3.0743	7.4146	< 0.001 ***

* $p < 0.05$.

** $p < 0.01$.

*** $p < 0.001$.

drospirenone, which is a quite high concentration. So, compared to our study, we could see that the percentage of females in stage IV declined from 80 % (SCt) to less (40 %-DRO1; 50 %-Hg1; Mix2–70 %; Mix3–20 %; Mix 4–30 %), when exposed to much lower concentrations of DRO (100 and 1000 ng L^{-1}). However, the average maturation stage of the ovaries did not change when exposed to DRO or Hg. Just at a higher

temperature, the effect was more visible. So, once again, these results demonstrate that the combination of stressors had a synergistic effect on the survival and gonads' maturation dynamics in the gastropod *N. lapillus*, since the combined effects of the three studied stressors were greater than the expected additive effect of the isolated stressors (according to Gunderson et al., 2016). In fact, from previous works, it was clear that the increase in temperature can exacerbate the negative effects caused by endocrine disruptors (e.g. Cardoso et al., 2017b, etc). These synergisms seem to be common in nature (Crain et al., 2008). However, some antagonistic responses can also be visible in other works. For example, Mannai et al. (2022) tested the effects of increased temperature and levonorgestrel (LNG) on the biochemical responses in the bivalve *Ruditapes decussatus* and observed that temperature diminished most of the responses to LNG. So, the responses can be variable.

Comparing the present work with Cardoso et al. (2018b), we could observe that drospirenone even at higher concentrations had a lower negative effect on *N. lapillus* gonads' maturation than a low concentration of levonorgestrel (10 ng L^{-1}) on zebrafish. So, drospirenone associated with increased temperature had a synergistic effect, being higher in females than in males. This pattern follows the literature, since high water temperature during pre-spawning phase may not be an impairment for normal gametogenesis in males (Miranda et al., 2013).

This study allowed us to infer that the last generation progesterone drospirenone has, potentially, a lower negative impact on the aquatic species than other progestins, like levonorgestrel, that even at environmental concentrations (10 ng L^{-1}) caused stronger negative effects in the reproduction of certain species, like zebrafish (Cardoso et al., 2017b).

Also, from this work, it is important to highlight the relevance of studying the effects of mixtures of chemicals in comparison with isolated factors, since the results can be different and, in nature, all living organisms are exposed to highly complex mixtures of anthropogenic chemicals. In this work, it was demonstrated that compounds, on their own, may not produce significant effects but can add up to elicit substantial mixtures' responses. The same was observed in previous works by Thrupp et al. (2018). Additionally, the mixture effect was even more pronounced at higher temperature having a synergistic response.

5. Conclusions

Our findings suggest that drospirenone and mercury, as isolated factors, can negatively affect the survival of the gastropod *N. lapillus*. This effect can be synergistic when these chemicals are mixed and under warming. Nevertheless, the tested treatments (including the different concentrations of both chemicals) did not cause any effect on the condition index and consumption rates of the model gastropod.

Additionally, both chemicals did not cause an evident effect on the maturation stage of ovaries and testis of the *N. lapillus* as single factors. However, in mixture and under higher temperature, it was clear a delay in the maturation stage of the ovary but not of the testis. However, both sexes evidenced a decline in the frequency of higher maturation stages to the detriment of less mature ones with the exposure to contaminants and higher temperature. So, our results suggest that the tested stressors had a higher negative impact on females than on males.

Thus, under a medium to long-term projected scenarios of warming, the constant exposure to a higher temperature and mixtures of the studied chemicals can trigger negative consequences on the survival of the species as well as on the dynamics of maturation of the female's gonads. This means that in the long-term, the species can become more fragile and the delay in the maturation of the gonads can affect, for example, the reproduction process. Since all the processes of the organism occur in a specific timing and are connected with what occurs in nature, if the timing of one process fails others can be compromised or vice-versa. According to Gunderson et al. (2016), the way organisms respond to multiple stressors depends greatly on the intensity and relative timing of each stressor. So, if the population of *N. lapillus* will be

affected, their prey can be affected too and consequently, modifications of whole trophic chain can occur. Therefore, the structure and functioning of intertidal rocky shore communities can be modified. So, the global ecosystem in which these gastropods are living can be compromised.

CRedit authorship contribution statement

H. Morais: Investigation, Software, Formal analysis, Writing – original draft. **F. Arenas:** Formal analysis, Software, Writing – review & editing. **C. Cruzeiro:** Methodology, Validation, Writing – review & editing. **S. Galante-Oliveira:** Conceptualization, Methodology, Writing – review & editing. **P.G. Cardoso:** Conceptualization, Methodology, Supervision, Writing – review & editing, Funding acquisition.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Patricia Cardoso reports financial support was provided by Fundação para a Ciência e Tecnologia. Patricia Cardoso reports a relationship with Fundação para a Ciência e Tecnologia that includes: employment and funding grants.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.marpolbul.2023.114841>.

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