Does thermal stress modulate the biochemical and physiological responses of *Ruditapes decussatus* exposed to the progestin levonorgestrel?

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**Abstract**

In this study, we investigated the effects of 1000 ng/l levonorgestrel (LNG) alone or combined with increased temperature of 20, 24, and 28 °C on the biochemical and physiological responses of the clam (*Ruditapes decussatus*) for 28 days. Our results revealed that female clams treated with levonorgestrel (LNG) alone showed enhancement of the antioxidant defense against oxidative stress related to the inductions of catalase (CAT), glutathione -S -transferase (GST), and protein sulfhydryl (PSH), while the elevated temperatures of 20, 24, and 28 °C diminished most of the specific responses to LNG and was the main factor in the determining the responses to combine exposures. The responses of lysosomal membrane stability, alkaline phosphatase, and NADP+-dependent isocitrate dehydrogenase detected were the most common signs of an adverse effect in all exposures. Female clams’ testosterone and estradiol responses to LNG were the most particular manifestations depending on the exposure. Overall, these findings showed clearly that chronic warming stress caused disruption in physiological, biochemical parameters of the female clam *R. decussatus*, and this may have implications for the whole organism and populations.

**Keywords** *Ruditapes decussatus* · Temperature · Levonorgestrel · Oxidative stress · Testosterone · Estradiol

**Introduction**

The Marine ecosystem is the final destination of various emergent contaminants including human and veterinary pharmaceuticals that have been detected in seawater, sediments, and in some cases also in tissues of marine organisms (Mezzelani et al. 2020; Mofijur et al. 2021). These findings have become an increasingly important concern in the assessment and protection of environmental risks. Among the pharmaceuticals released into the marine ecosystem, there are oral contraceptives and other hormonal drugs that affect the reproductive performance of non-target organisms via the disruption of sexual maturation and sexual differentiation (Leonard et al. 2014; Almeida et al. 2020).

The adverse physiological effects of these compounds and their metabolites arise from their high bioactivity and their persistence in the environment (Oropesa and Guimaraes 2021; Rodrigues et al. 2021). In addition, the mechanism of action of hormones in oral contraceptives, by which the reproduction of non-target organisms is impaired, is considered to be similar to its intended therapeutic use in humans (e.g., hormone receptor-mediated response) because many
therapeutic targets are highly conserved across the species (Gunnarsson et al. 2008).

Levonorgestrel (LNG) is one of the synthetic progestins commonly used in birth control pills and has been considered a potentially harmful substance that has been detected at a level of 1 to 200 ng/L in the marine ecosystem (Fent 2015; Cardoso et al. 2017; Oropesa and Guimarães 2021). Several studies have reported that levonorgestrel can act as a potent endocrine disruptor, even at ng/L levels, ultimately causing adverse effects in fish (Runnalls et al. 2013; Cardoso et al. 2019; Thrupp et al. 2018), crustaceans (Furuhagen et al. 2014), amphibians (Säfholm et al. 2016), and bivalves (Contardo-Jara et al. 2011). The real danger of LNG for the reproduction of aquatic organisms is related not only to the structural similarity of endogenous progesterone but also to its interaction with other steroid hormone receptors and thus exerts a mixture of hormonal effects. Thus, levonorgestrel interacts not only with progesterone receptors (PR) but also with androgen (AR), estrogen (ESR), glucocorticoid (GR), and mineralocorticoid (MR) receptors (Fent 2015) leading to endocrine disruption and impaired reproduction in non-target organisms (Frankel et al. 2016; Cardoso et al. 2018; Säfholm et al. 2016). In this regard, environmental residues of LNG may pose potential risks to aquatic organisms. Svensson et al. (2013) found strong androgenic effects of LNG in female three-spined stickleback (Gasterosteus aculeatus). Moreover, downregulation of follicle stimulating hormone, aromatase, estrogen receptors, and androgen receptors gene expressions was observed in fathead minnow larvae exposed to LNG (Liang et al. 2015). Others hypothalamic-pituitary-thyroid axis genes were highly upregulated in zebrafish larvae exposed to LNG (Li et al. 2015).

Other aspects of the toxicity of levonorgestrel are linked to its transformation into active metabolites which interact with endogenous hormone receptors causing disruption of endocrine regulation of reproduction (Besse and Garric 2009). It was demonstrated that the 3β-5α-tetrahydroderivatives from LNG exhibit oestrogenic activity (García-Becerra et al. 2002).

Temperature is one of the most important environmental factors that control physiological processes, metabolic functions, growth, and reproduction of several marine invertebrates, including bivalves (Amorim et al. 2020; Louis et al. 2020; Hamdani et al. 2020; Pouil et al. 2021). For example, the reproductive cycle of the bivalve R. decussatus is strongly dependent on seawater temperature (Matias et al. 2009, 2016) and the optimum temperature is around 20 °C (Delgado and Camacho 2005). Over the past century, global warming has become harmful and even dramatic (IPCC 2014; Guiot and Cramer 2016). Numerous studies have reported the potential negative impact of increased temperatures on bivalves, such as changes in their respiratory rate (Jansen et al. 2009), their metabolism (Anestis et al. 2007) and their immune responses (Malagoli et al. 2007), altering sexual maturation (Martínez and Pérez 2003), reducing survival (Arnberg et al., 2013), inducing abnormal larval development, and reducing fertility and growth of larvae and juveniles (Nguyen et al. 2012). The repeated exposure of pearl oysters to simulated marine heatwaves enhances the thermal tolerance by various strategies such as less costly energy-utilizing to compensate for thermal stress induced physiological interferences (He et al. 2021).

The simultaneous multiple stressors like elevated temperature and pollutants can constitute a real danger for the marine organism’s health as pollutants can be strongly modified by warming seawater and the organism’s sensitivity to pollutants can be affected by heat stress (Costa et al. 2020; Freitas et al. 2020). In addition, pollutants can damage the ability of organisms to respond to rapid increases in temperature (Hooper et al., 2013; Noyes and Lema 2015). It has also been shown that heat stress can exaggerate the toxic effects of chemicals through several mechanisms by modifying their bioavailability, absorption, and transformation, by increasing mitochondrial damage and oxidative stress, by altering physiological capacities and by causing an energy imbalance (Izagirre et al. 2014).

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Compared to the many studies on the combined effects of heat stress and heavy metals on marine bivalves, knowledge about the interactive effects of synthetic progestins such as levonorgestrel and elevated temperature is much rarer, although its potential impacts could be more important and with unintended consequences. For example, studies of Cardoso et al. (2017) and (Cardoso et al. 2019) have already demonstrated the complex effects of the combined LNG and elevated temperature on the reproduction success and on the ovary and liver structure and function of zebrafish (Danio rerio) (Cardoso et al. 2017, 2019).

The clam (R. decussatus), a benthic filter feeder bivalve, is widely distributed along the Mediterranean coast and considered a valuable seafood and one of the most important bivalve species in the economy of Europe’s southern countries, namely Italy, Spain, and Portugal (Matias et al. 2009). On the Tunisian coast, R. decussatus is particularly abundant in the Gulf of Gabès, and represents 94% of the overall exploitation of shellfish in Tunisia (C.G.P., 1996). This resource of great economic value gives the present study a certain importance. The R. decussatus clam has been widely used as a preferred model animal in ecotoxicology to assess contamination of the marine ecosystem due to its sedentary, ease of collection, wide geographic distribution, and accumulation of large contaminant capacity from seawater and sediment (Jebali et al. 2007; Sellami et al. 2014). Therefore, it is of great interest to understand the effects of combined exposure to heat stress and levonorgestrel on the physiological and biochemical responses of the R. decussatus clam in order to predict their negative impact in the
context of winter-summer seasonal change or in the context of global warming and synthetic hormones exposure.

In the present study, the clam *R. decussatus* was used to investigate the combined chronic effects of the synthetic progestin LNG and increasing temperatures of 20 °C, 24 °C, and 28 °C on its physiological and biochemical performances and on the gonad histological structure. We were particularly interested in the assessment of responses of oxidative stress biomarkers, hormonal levels, and gonadal tissue characteristics of female clams (*R. decussatus*) exposed to LNG and heat stress for 28 days.

**Materials and methods**

**Clam collection and experimental design**

Three hundred specimens of *R. decussatus*, with shell length between 28 and 45 mm, were collected on a reference site of the Tunisian coastal zone (Hamida et al. 2004). Then, they were transported to the laboratory where they were acclimatized for 7 days before the experiments. The clams were maintained in 20 l aquariums containing continuous aerated seawater (salinity of 38.025± 2 mg/l, temperature of 20 ±0.5 °C, pH 8.2) and fed with microalgae (*Isochrysis* galbana) twice a week (1.109 cells/animals). The seawater was renewed every 48 h.

After acclimatization and in order to assess the effect of exposure to LNG, under increasing temperatures, the clams were randomly divided into six experimental groups of fifty specimens each and placed in 20-L glass aquariums with seawater continuously aerated and supplied with microalgae. We note that true replicates were not used in each treatment:

- **Group 1 (T20-):** clams exposed to the ambient temperature of 20 °C ± 0.5 °C and 0 ng/l of LNG (control condition);
- **Group 2 (T20+):** clams exposed to the ambient temperature of 20 °C±0.5 °C and 1000 ng/l LNG;
- **Group 3 (T24-):** clams exposed to temperature of 24 °C±0.5 °C and 0 ng/l LNG (warming condition);
- **Group 4 (T24+):** clams exposed to temperature of 24 °C±0.5 °C and 1000 ng/l LNG (condition of combined stresses of warming and LNG contamination);
- **Group 5 (T28-):** clams exposed to temperature of 28 °C±0.5 °C and 0 ng/l LNG (extreme warming condition);
- **Group 6 (T28+):** clams exposed to temperature of 28 °C±0.5 °C and 1000 ng/l LNG (condition of combined stresses of extreme warming and LNG contamination).

Every 2 days, the seawater was renewed and fresh LNG was added at the desired concentration. The seawater temperatures of the different clam groups were maintained stable using electronic thermostats. The clams were maintained under experimental conditions for 28 days. The LNG concentration of 1000 ng/L was higher than the environmental concentration and was selected based on the several laboratories works such as Svensson et al. (2013), Svensson et al. (2014), Cruzeiro et al. (2019) and Cardoso et al. 2019. The temperature range was chosen to mimic the seasonal changes of the seawater temperature that varied on the Tunisian littoral from 18 °C in winter to 28 °C in summer (Kamel et al. 2014). After the exposure time, the total weight and soft tissues of each male and female clam were measured for the condition index (CI) determination. Then, twenty female clams per treatment were used for physiological parameters determinations including hormonal and biochemical analysis, vitellogenin like proteins (VTG) level, and gonad histology. The male clams were not used in this work.

The hemolymph (about 1.2–1.8 ml per clam) samples were collected from the anterior adductor muscle with a 1-ml plastic syringe and stored in ice for lysosomal membrane stability and vitellogenin like proteins (VTG) level determinations.

The gonad tissues of twenty female clams were carefully removed and seven of them divided into two parts: the first part was placed in the Davidson solution for histological study, whereas the second part was stored at −80 °C for steroid levels determination. The digestive gland was dissected and stored at −80 °C until biochemical parameters measurements.

**Biochemical parameters determinations**

Biochemical parameters (superoxide dismutase (SOD), catalase activity (CAT), glutathione S-transferases activity (GST), alkaline phosphatase activity (ALP), isocitrate dehydrogenase activity (ICDH), lipid peroxidation levels (thiobarbituric acid reactive substances; TBARS), conjugated dienes (CoD), and protein sulfhydryl levels (PSH)) were measured in the digestive gland of the control and treated clams. Each parameter was measured in individual clam and in duplicate. Digestive gland was homogenized (1/4, w/v) in ice-cold phosphate buffer (0.1 M, pH=7.4) and the obtained homogenate was then centrifuged at 10,000 × g for 20 min at 4 °C. The pellet was discarded and the supernatant was collected, divided into aliquots, and stored at −80 °C for biochemical parameters analysis (SOD, CAT, GST, ALP, ICDH, TNARS, CoD, and PSH). The quantities of proteins present in supernatant were measured spectrophotometrically at 595 nm (Bradford 1976), using the bovine serum albumin as the standard.
Oxidative stress biomarkers

Catalase activity (CAT) was determined according to Claiborne (1985) by following the decrease of absorbance at 240 nm due to H₂O₂ decomposition. The reaction mixture (final volume of 1 ml) contained 890 μl 0.1M phosphate buffer (pH 7.5), 100 μl of hydrogen peroxide H₂O₂ (0.5 M), and 10 μl of supernatant. CAT activity was evaluated using a spectrophotometer (240 nm). Results were expressed as μmol hydrogen peroxide transformed/min/mg protein.

Superoxide dismutase activity (SOD) was determined by the inhibition of pyrogallol autoxidation in the presence of a superoxide generation system at 420 nm (Marklund and Marklund 1974). After the addition of cold ethanol: chloroform (200 μl, 1:1) to 250 μl of supernatant fraction, the mixture was centrifuged at 2500×g for 25 min at 4 °C. The supernatant was recovered and used to determine SOD activity in the presence of pyrogallol. The enzymatic activity was expressed in μmol/min/mg of protein. One unit represents the amount of SOD, which inhibits 50% of pyrogallol autoxidation.

Glutathione S-transferase (GST) activity was quantified according to Habig et al. (1974), following the conjugation of glutathione with 1-chloro-2,4-dinitrobenzene (CDNB) et 340 nm for 2 min. The reaction mixture contained cytosolic protein, CDNB (1 mM), glutathione reduced form GSH (4 mM), and sodium phosphate buffer (100 mM, pH 7.5). Results were expressed as μmol GSH-CDNB produced/min/mg protein.

The lipid peroxidation level was evaluated by the contents of thiobarbituric acid reactive substances (TBARS) and conjugated dienes (CoD). Thiobarbituric acid reactive substances concentration was determined via a color reaction with thiobarbituric acid reagent (Buege and Aust 1978). The reaction was assessed at 532 nm. Results were expressed as nmol TBARS / mg protein. Conjugated dienes are the primary lipid peroxidation products of polyunsaturated fatty acids and were determined by the method of Recknagel and Ghoshal (1966). One hundred microliters of tissue homogenate was transferred to a chloroform/methanol mixture (2:1). The whole mixture was vortexed and centrifuged at 2500×g for 10 min. After evaporation, the extract was redissolved in 1 ml cyclohexane and the absorbance was measured at 233 nm. Results were expressed as nmol/mm protein.

Protein sulfhydryl (PSH) content was carried out based on the method of Grattagliano et al. (1996) and described previously by Amri et al. (2020). Briefly, the PSH content was determined by subtracting the absorbance of non-sulfhydryl proteins (A2) from absorbance of the total protein sulfhydryl (A1) in the liver supernatant. The concentration of total protein sulfhydryl was determined using 5,5-dithio-bis-2-nitrobenzoic acid (DTNB; 0.01 M) and the absorbance was measured at 412 nm (A1). Subsequently, the protein sulfhydryl (PSH) was precipitated using 10% TCA and then centrifuged. The clear supernatant containing non-sulfhydryl proteins was treated with 5,5-dithio-bis-2-nitrobenzoic acid (DTNB; 0.01 M), which was reduced to yellow TNB (5-thiobis (2-nitrobenzoic acid)) and measured at 412 nm (A2). PSH content was expressed as μg PSH/mg of protein.

Alkaline phosphatase activity

Alkaline phosphatase activity (ALP) was measured using the spectrophotometric method of Nemec and Socha (1988). Enzyme activity was determined in Tris–HCl buffer (0.1 M, pH 8.6) and the substrate p-nitrophenylphosphate (PNPP 5 mM). This mixture was incubated for 30 min at 30 °C. The reaction was stopped by addition of 1 ml of sodium hydroxide (NaOH, 0.5 M) after incubation. The absorbance was measured at 405 nm on a spectrophotometer. Results were calculated in terms of nmoles p-nitrophenol /min/mg protein.

Isocitrate dehydrogenase activity

Isocitrate dehydrogenase activity (ICDH) was determined according to Berna and Bergmeyer and Bernt (1974). The enzymatic activity was measured by using nicotinamide adenine dinucleotide phosphate (NADP⁺) and isocitrate as substrate. ICDH activity was monitored by measuring the reduction of NADP⁺ to NADPH, which has an extinction coefficient of 6.22 ml/cm mmol at 340 nm.

Lysosomal membrane stability assay

Lysosomal membrane stability was evaluated by the neutral red retention time (NRRT) assay as a marker of bivalve cellular stress according to Lowe and Pipe (1994) and Ruiz et al. (2019) with some modifications. At the end of the experimental exposure, 0.2 ml hemolymph was withdrawn from each clam adductor muscle using a 2-mL syringe with 0.2 ml of saline solution (4.77 g/l HEPES, 25.48 g/l NaCl, 13.06 g/l MgSO₄, 0.75 g/l KCl, and 1.47 g/l CaCl₂) to a final volume of 1 L reverse osmosis water; pH 7.3). Five clams per group were used for this test. Thirty microliters of hemolymph saline mixture was transferred into a microscope slide and left in humidity chamber for 15 min at room temperature to allow the cells to adhere. The cells then were incubated in the following treatment solutions. A dye stock solution was prepared by dissolving 28.8 mg Neutral Red (Sigma-Aldrich) in 1 mL of dimethyl sulfoxide. A working solution was then prepared by diluting 2 μL of stock solution with 1 mL of physiological saline solution. Neutral red working solution (30 μL) was added to the area containing the attached cells and a cover slip was applied. After 15 min of incubation, excess dye was washed out, and slides...
were sealed with a coverslip and observed under an optical microscope. Hemocytes were microscopically examined at 15 min intervals for up to 90 min, to evaluate the time at which 50% of cells had leaked to the cytoplasm the neutral red previously trapped by lysosomes.

### Endogenous steroid hormones analysis

Testosterone and oestradiol levels were measured in female gonad tissues of the control and treated clams as described by Hiroi et al. (1997) and by Poursaeid et al. 2012. Briefly, gonad tissue samples were homogenized with distilled water. Then, 300 μL of the homogenized tissues was transferred to new tubes and 3 ml of diethylether was added and shaken vigorously for 2 min. After freezing at – 80 °C, the top layer containing diethylether was collected and dried at room temperature. Dry extracts were resuspended in a potassium phosphate buffer (50 mM, pH=7.4) and assayed for testosterone and estradiol concentration using commercial RIA kits.

### Determination of vitellogenin like proteins

Levels of vitellogenin-like proteins were determined in hemolymph of both the control and treated clams by an organic alkali-labile phosphate assay following by the method of Blaise et al. (1999). This assay is based on the extraction of the lipophosphoproteins in ether followed by subsequent alkali treatment for labile phosphate release that can be quantified by the method of Stanton (1968). Briefly, 500 μl of hemolymph was mixed with 500 μl of t-butylmethylether for 30 min at room temperature. These emulsions were mixed by a vortex agitator 3 times during the extraction period. A 400-μl volume of the organic phase was carefully transferred to another microcentrifuge tube containing 100 μl of 1M NaOH for 60 min at 50 °C to allow hydrolysis of phosphate bound. The levels of free phosphates were determined in the aqueous phase according to the phosphomolybdenum method of Stanton (1968). For inorganic phosphate standard curve, increasing KH2PO4 concentrations were prepared. Results were expressed as μg PO4/mg protein. Protein concentrations in hemolymph were quantified according to Bradford (1976).

### Condition index determination

The condition index (CI) is an interesting indicator of clam general physiological status. The CI of the control and treated clams was measured according to Lobel et al. (1991); CI = (fresh weight of soft tissues/total weight) * 100.

### Histological analysis

After dissection, gonadal tissues from unexposed and exposed clams were carefully excised and immediately fixed in Davidson’s solution for 48 h. The tissues were then dehydrated in a series of alcoholic solutions of increasing concentration (from 70 to 100%), then clarified in xylene and embedded in paraffin. Five-micrometer thin sections were obtained using a rotating microtome and then stained with hematoxylin and eosin before observation under an optical microscope coupled to an image acquisition device. The histological sections obtained were used for the determination of the sex and the number of oocytes. The photos of the gonad sections were analyzed by Image J software and the number of mature oocytes per visual field was determined.

### Statistical analysis

Statistical analyses were performed using XLSTAT 2014 software. For the biochemical (CAT, GST, SOD, ICDH, ALP, PSH, TBARS and CoD), physiological (Estradiol, Testosterone, Number of Oocytes, Condition index and vitellogenin-like proteins), and lysosomal membrane stability responses, one-way ANOVAs were carried out to test for differences among the treatments. The 2-way ANOVA (temperature x LNG) was also applied to detect differences among treatments. Prior to ANOVA, the data were tested for normality and homogeneity of variance assumptions. Differences were considered statistically significant at p < 0.05. Different letters a, b, c, and d indicated significant differences between groups.

### Results

#### Biochemical responses

Clams treated with LNG for 28 days at an ambient temperature showed a significant increase in catalase (CAT) (p <0.05; Fig. 1A) and glutathione S transferase (GST) activities (p < 0.05; Fig. 1C) about 2.2-fold and 1.9-fold (p < 0.05) when compared to the control. The superoxide dismutase activity (SOD) (Fig. 1B), lipoperoxidations level expressed by the thiobarbituric acid reactive substances (TBARS, Fig. 1D) and conjugated dienes levels (Fig. 1E) were not significantly changed. The protein sulfhydryl (PSH) level (Fig. 1F) and alkaline phosphatase activity (Fig. 1G) were enhanced 2.7-fold (p < 0.05) and 4-fold (p < 0.05), while the isocitrate dehydrogenase activity (ICDH) (Fig. 1H) was reduced 2-fold (p < 0.05) when compared to the control. 2-way ANOVA showed that clams exposed to LNG significantly altered CAT, GST, ALP, ICDH activities, and TBARS levels (Table 1).
The clams exposed only to various temperature degrees (20 °C, 24 °C, and 28 °C) exhibited alteration of biochemical parameters marked by a high reduction of CAT ($p < 0.05$; Fig. 1A) and GST activities ($p < 0.05$; Fig. 1C). In contrast, SOD activity was enhanced in an increased temperature-dependent manner. The SOD activity increased about 1.37-fold ($p < 0.05$) at 24 °C and 1.84-fold ($p < 0.05$) at 28 °C when compared to the relative control which was maintained at 20 °C. The PSH level was increased 6.49-fold ($p < 0.05$) in clams exposed to 24 °C and 6.68-fold ($p < 0.05$) at 28 °C. The thiobarbituric acid reactive substances (TBARS, Fig. 1D) and conjugated dienes levels (Fig. 1E) were significantly increased in clams exposed to 24 °C alone or to elevated temperature combined to LNG 24 °C+LNG and 28 °C+LNG where the TBARS increased about 1.85-fold, 2.22-fold, and 1.63-fold ($p < 0.05$). The ICDH activity was significantly ($p < 0.05$) inhibited in clams exposed to 24 °C as opposed to clams exposed to 28 °C (Fig. 1H). An increased temperature of 24 °C significantly increased the alkaline phosphatase activity compared to the control (Fig. 1G). 2-way ANOVA demonstrated that clams exposed to the elevated temperature of 20, 24, and 28 °C significantly altered the biochemical responses (CAT, SOD, GST, ALP, ICDH, PSH, TBARS, and CoD) (Table 1).

Clams exposed to LNG +24 °C and to LNG + 28 °C showed a decrease ($p < 0.05$) in CAT and GST activities when compared to the control. However, no significant differences were observed when compared to the clams exposed only to 24 °C or to 28 °C. SOD activity and PSH levels were increased ($p < 0.05$) while ICDH activity was reduced ($p < 0.05$) in treated clams with the exposure to LNG +24 °C and LNG + 28 °C in comparison to the control. The thiobarbituric acid reactive substances (TBARS, Fig. 1D) and conjugated dienes (Fig. 1E) levels were enhanced in treated clams with LNG +24 °C and LNG + 28 °C. The highest conjugated dienes levels were observed in clams exposed to LNG + 28 °C where the increase was about 1.53-fold when compared to the clams exposed to 28 °C ($p < 0.05$). The 2-way ANOVA showed that exposure to combined LNG and increased temperatures caused significant effect on the CAT, GST, TBARS, PSH, and ICDH levels. The PSH and CoD levels were not influenced by the exposure to LNG;
however, its responses were modulated by the exposure to the elevated temperature and to the combined temperature and LNG (Table 1).

**Lysosomal membrane stability assessment**

Lysosomal membrane integrity of hemocytes was evaluated in clams treated with levonorgestrel and an elevated temperature for 28 days by the measurement of neutral red retention time (NRRT) as biomarker of subcellular damage of LNG and heat stress. A significant diminution ($p < 0.05$) of the NRRT about 18.42% was observed in clams exposed to LNG at ambient temperature of 20 °C when compared to the control group. High reductions ($p < 0.05$) of NRRT were observed in clams coexposed to the levonorgestrel and heat stress (LNG+ 24 °C and LNG+ 28 °C (Fig. 1I). 2-way ANOVA showed that exposure to LNG or to the increased temperatures significantly affected lysosomal membrane stability (Table 1).

**Physiological responses**

**Gonad steroids concentrations**

Our results indicated that females treated with LNG exhibited a significant high gonad estradiol level (2.23-fold) ($p$
and without changes in testosterone levels (Fig. 2A). The exposure to the heat stress (24 and 28 °C) induced a decrease of estradiol and testosterone levels with significant \( p < 0.05 \) diminution of testosterone at 28 °C. Clams exposed to LNG + 24 °C exhibited no changes in estradiol level while the testosterone level was significantly low \( p < 0.05 \). A non-significant decrease of estradiol and testosterone levels was recorded in clams co-exposed to 28 °C + LNG compared with the control group. 2-way ANOVA showed that exposing clams to increased temperatures alone or in combination with LNG caused a significant effect on estradiol and testosterone levels.

### Determination of vitellogenin like proteins: ALP assay

Females exposed to the temperatures only (24 and 28 °C) showed a decrease in the vitellogenin like proteins levels when compared to the control clams (Fig. 2C). However, no change in vitellogenin like proteins levels was observed in clams exposed to LNG + 20 °C. The co-exposure to the heat stress and levonorgestrel (LNG +24 °C and LNG + 28 °C) induced a significant diminution of vitellogenin like proteins levels \( p < 0.05 \; \text{Fig. 2C} \). 2-way ANOVA showed that exposure of clams to the increased temperature caused significant effect on the VTG level, it was not modulated by the exposure to the LNG neither to the combined temperature and LNG (Table 1).

### Condition index

The condition index of the control and treated clams (R. decussatus) with LNG and temperature for 28 days was not much affected except clams contaminated with LNG at 24 °C when compared to the control (Fig. 2D).

### Histological analysis

Control female gonads were characterized by normal gonadal tubules with many free mature spherical oocytes occupy the lumen (Fig. 3A). Exposure to LNG inhibited gonadal development and significantly decreased the number of oocytes per follicle than those of the control (Fig. 3B). The decrease in the number of oocytes per follicle area was approximately (58%) compared to the control group. The increase in temperature (24 °C) had a positive effect on maturation of female clams. Results depicted in Fig. 3C clearly showed that exposure to 24 °C enhanced maturation of females via a significant increase number of oocytes within follicles (about 1.29-fold) compared with control group. However, gonads of clams exposed to 28 °C showed a complete absence of oocytes (Fig. 3D). In exposed females to the combined LNG + 24 °C, atrophied follicles and the central lumen were nearly empty, only a few residual oocytes were...
found at the periphery of the follicles (Fig. 3E). Similarly, coexposed females to the increasing temperature and levonorgestrel (LNG+ 28 °C) showed empty (0 oocytes per follicle) and atrophied of ovarian follicles. 2-way ANOVA showed that exposure to combined LNG, to the increased temperature or to the combined temperature and LNG caused significant effect on the number of gonad oocytes (Table 1).

Figure 3F showed also an increase in the inflammatory response characterized by a focal or diffuse hemocytic infiltration and formation of hemocytic aggregates (granulocytomas) in the connective tissue.

Discussion

Biochemical responses

Synthetic progestins in the marine ecosystem have adverse effects on the marine sedentary organisms like bivalves even
at very low concentration of ng/l. Abundant studies are available on the potential endocrine disruption effects, reproduction alteration of the synthetic hormones, and the physiopathology mechanisms in non-target organisms (Fent 2015; Oropesa and Guimarães 2021). Moreover, the interactive effect of the increased temperature and a synthetic progestin such as LNG has received major attention (Ward et al. 2020; Cox Megan 2016; Sieratowicz et al. 2011). In this study, is addressed the combined effects of the increased temperature of 20, 24, and 28 °C and a synthetic progestin LNG on the physiological and biochemical responses of clam (R. decussatus). The nominal concentration 1000 ng/L of LNG could be changed by adsorption of the compound to the aquaria and silicone tubings. Several authors showed clearly that measured LNG concentrations were lower than the nominal and the diminution level depends on the exposed concentrations (Zeilinger et al. 2009; Svensson et al. 2013, 2014). The same authors reported that the concentrations were stable during the course of the experiment and the measured concentrations would be essential for the data interpretation.

Unfortunately, in this work, we did not measure the LNG concentrations in aquaria seawater; for this reason, we considered the nominal concentration of LNG for the representation and the interpretation of the results.

The exposure to the LNG exhibited marked oxidative stress generation and we noted a significant increase of catalase (CAT), glutathione-S-transferase (GST) and protein sulphydryl (PSH) levels. The enhanced antioxidative...
defence prevented the formation of lipid peroxidation products, such as lipid hydroperoxides and their derivatives (Doyen et al. 2005). In this study, higher GST and CAT activities in contaminated clams by LNG were associated with the conjugated dienes (CoD) and thiobarbituric acid reactive substances (TBARS) levels correspondent to the control which may indicate the capacity of organisms to eliminate the excess of ROS and prevent membrane lipid peroxidation. Moreover, the increase of GST activity hints on biotransformation of phase I activated LNG metabolites as an immediate effort to facilitate excretion. The effects of levonorgestrel on the oxidative stress parameters at molecular genes expressions were examined in mussels (Dreissena polymorpha), and a marked alteration of mRNA was observed in several genes belonging to metabolism, antioxidative defense, and protein damages (Contardo-Jara et al. 2011).

Temperature is one of the most important environmental factors that control biochemical physiological parameters, growth, reproduction, and survival of the sedentary bivalves including clams (R. decussatus). However, many studies reported that exposure to the post preferred temperature induced oxidative stress (Hu et al. 2015; Freitas et al. 2019; Rahman et al. 2019).

In the present study, the exposure of clams to the graded temperature of 20, 24, and 28 °C induces oxidative stress marked by increased SOD and PSH levels to avoid oxidative damage to cellular macromolecules including lipids. It is well known that heat stress caused by an elevated temperature constitutes a principal challenge to marine organisms, affecting their metabolism (Louis et al. 2020; Li et al. 2021) which can make them more sensitive to chemical exposure. Conversely, chemical exposure may affect the organism’s ability to cope with thermal stress (Nardi et al. 2018; Costa et al. 2020). Our results revealed that the combined effect of LNG and a higher temperature of 28 °C caused a high lipoperoxidation marked by elevated TBARS production. These results may be indicating the possibility of the enhanced metabolic rate in clams kept at a higher temperature, which induce an increase in the seawater filtration rate and, consequently increased LNG uptake. Higher contaminants uptake due to increased temperatures have been reported by many authors (Grasset et al. 2016; Hani et al. 2018; Boukadida et al. 2017). Moreover, the increased reactive oxygen species (ROS) production under co-exposure to LNG +28 °C for a long period of 28 days caused inability of organisms to eliminate the excess of ROS and prevent membranes cellular damage by antioxidative and detoxifying enzymes.

Other biochemical parameters alkaline phosphatase (ALP) and NADP-dependent isocitrate dehydrogenase (ICDH) were affected by exposure to levonorgestrel and temperature. Alkaline phosphatase is a hydrolase enzyme responsible for transphosphorylation and has an important role in the general energetics of an organism. ALP is involved in a variety of metabolic processes, such as molecular permeability, protein synthesis, growth and cell differentiation, gonadal maturation, and steroidogenesis (Ram and Sathayanesan 1985). Although this enzyme has been considered as a useful biomarker because of its sensitivity to a variety of pollutants such as trace metals (Zhou et al. 2021), nanoparticles (Ray et al. 2020) poly-aromatic hydrocarbons xenobiotic (Mansour et al. 2017), and many others xenobiotic (Ranilaltha et al. 2014; Nandurkar 2017), very little data focused on the impact of levonorgestrel and heat stress on the ALP activity. In this work, ALP activity was increased in response to LNG alone and to LNG associated with thermal stress. Similar increased ALP activity was observed by Hu et al. (2015). In this study, clams co-exposed to LNG and heat stress showed increased ALP activity compared to the control. Hansen et al. (1992) explained that increases in ALP activity resulting in changes of metabolic activity allowed adaptation of organism to submitted stress. From a physiological perspective, alkaline phosphatase (ALP) is involved in the calcium absorption and amorphous calcium carbonate (ACC) formation. The enzyme is affected by heat stress and is considered to be a marker for biominalization activity in mollusks. The expression level of ALP gene and its activity are both downregulated under exposure to elevated CO2 and temperatures in the pearl oyster (Pinctada fucata) (Li et al. 2016). The authors considered that significant changes of the downregulation of net calcification rate are strongly responsive to increased CO2 and temperatures in marine pearl oyster (Li et al. 2016).

NADP-dependent isocitrate dehydrogenase (ICDH) is an essential enzyme that catalyzes oxidative decarboxylation of isocitrate to a-ketoglutarate in Krebs pathway. A primary function of NADP+-dependent isocitrate dehydrogenase is the control of cytosolic and mitochondrial redox balance and antioxidative defense by supplying reduced NADP+ for antioxidative systems (Yang et al. 2002). In the present study, we revealed a significant decrease of ICDH activity in clams exposed to LNG only and to LNG associated with temperatures (24 and 28 °C). The ICDH activity inhibition can be due to the chronic exposure of clams during the 28 days to oxidative stress and as a consequence a decrease in its capability to deal with high oxygen reactive species generation. The NADP+-dependent isocitrate dehydrogenase (ICDH) role is essential to regenerate cellular NADPH and as consequentially maintain the cell redox balance (Oliveira et al. 2018). Its inhibition may also indicate decrease of the ability to deal with oxidative stress, as also suggested by the inhibition catalase and glutathione S transferase activities and significant increase of lipid peroxidation levels. The ICDH activity inhibition was previously reported in freshwater bivalve Corbicula fluminea exposed to microplastics (0.13 mg/L), mercury (30 μg/L), and to a mixture of both substances. This is particularly critical because mercury and
microplastics induced oxidative stress as indicated by the significant increase of antioxidant activities, and may have contributed to the failure of the antioxidant system to prevent oxidative damage to occur as indicated by the significant increase of lipid peroxidation (Oliveira et al. 2018).

**Lysosomal membrane stability assessment: subcellular biomarker of toxicity**

Lysosomes are intracellular organelle containing various hydrolytic enzymes associated with endogenous macromolecules and chemicals catabolism and metals sequestration (Moore et al. 2007). Lysosomal membrane is target of several environmental stress that provokes diminution of its integrity (Shaw et al. 2019; Parisi et al. 2021). Therefore, its usefulness in several biomonitoring exercises is explained by its nondestructive biomarker aspect and easeful measurement by the neutral red retention time assay (NRRT) (Lowe and Pipe 1994). A significant reduction in lysosomal membrane stability was noted in clams exposed to levonorgestrel alone and the reduction was more accentuated with combined exposure to the LNG and temperatures (24 and 28 °C). These results suggested also that oxidative stress generated by LNG and thermal stress could be one of the toxic ways that altered the lysosomal membrane integrity cell injury.

It is well known that, lysosomal membrane is target of several environmental stress that provokes diminution of its integrity (Shaw et al. 2019; Parisi et al. 2021). In the study, a significant reduction in lysosomal membrane stability was noted in clams exposed to LNG alone or in combination with the increasing temperatures (24 and 28 °C). The seawater warming is predicted to affect marine ecosystems at different levels of biological organization. Elevated temperature can enhance oxygen consumption in clams and therefore may rapidly produce reactive oxygen species (ROS), resulting in oxidative stress affecting the lysosomal membrane permeability, protein sulphhydril levels, alkaline phosphatase (ALP), and isocitrate dehydrogenase (ICDH) activities. Moreover, the chronic exposure of clams to temperature stress can facilitate the leakage of lysosomal hydrolytic enzymes into the cell internal environment leading to various subcellular damage from proteolysis, lipolysis, and digestion process disruption to cell death (Lowe et al. 1995; Xue and Renault 2000).

**Physiological responses**

Endogenous gonads hormones including 17β-oestradiol, testosterone, and progesterone have been reported in many marine bivalves playing crucial role in the regulation of reproductive processes, as found in vertebrates (Zhu et al. 2018; Osada and Matsumoto 2020). Many vertebrate sex steroids have been identified in invertebrate (Juner and Porte, 2007). It also has been documented that vertebrate sex steroids are involved in gonadal and gamete development in bivalves (Croll and Wang 2007; Scott 2013). Therefore, several authors including Giusti and Joaquim-Justo (2013), Blalock et al. (2018) and Ojoghoro et al. (2021) reported that exposure of bivalves to natural steroids and to endocrine disrupting chemicals has been reported to induce adverse effects on invertebrate reproduction, mainly by hormone binding directly to steroid receptors or altering hormone metabolism, similar to that in vertebrates.

One of the primary functions of endogenous gonads hormones is related to the gonadal development and sexual maturation during the reproductive cycles. The exposure of the adult female clams (*R. decussatus*) during the reproduction moment to levonorgestrel increased the oestriadiol level about 2.23-fold. This clear increase of estradiol is contrary to the results of most of the previous studies in fish, which mainly reported the decrease of estradiol by LNG exposure (Kroupova et al. 2014; Runnalls et al. 2013). In the present study, the increased estradiol level was associated with diminution of the oocytes number and with the vitellogenin like proteins level. Based on these results, our hypothesis is that LNG and its metabolites 5α-LNG, 3α,5α-LNG, and 3β,5α-LNG could mimic estradiol and bind to its receptor and inhibit the gonad development, oocyte maturation, and vitellogenin like proteins synthesis. This hypothesis is supported by García-Becerra et al. (2002), Svensson et al. (2013), Frankel et al. (2017), and Cardoso et al. (2017).

Vitellogenin like proteins (VTG) are glycoprophospholipoproteins synthesized by mature gonads of bivalves and stored in developing oocytes. It has been shown that vitellogenin like proteins under control of 17β-oestradiol were interacting with the estrogen receptor (Osada et al. 2003). Our results indicated that clams exposed to LNG only for 28 days did not show changes in hemolymph VTG level when compared to the relative control. These results are in part corroborated by the observations of Zacchi et al. (2014) which detected a downregulation of vitellogenin like proteins in the zebrafish liver when exposed to the synthetic progesterone drospirenone or in combination with progesterone for 14 days. Frankel et al. (2017) also demonstrated that exposure to 100 ng/L LNG caused a decrease in the blood plasma vitellogenin levels in females of *Pimephales promelas*. Vitellogenin like proteins are usually considered a good biomarker to detect estrogenic contamination and are also elicited by aromatized androgens, in vitro (Kim and Takemura 2003) and in vivo (Madureira et al. 2018). In our case, this decrease may be also be explained by the binding of LNG to VTG receptor causing blocked estrogen binding to this receptor or it can be associated with the androgenic activity of LNG (Hua et al. 2015). Besides, it can be explained by LNG interfering with the release of nervous system releasing neurohormones to
Temperature has a crucial role in the modulation of the bivalve's gonads development and gametes maturation (Villanueva-Gutiérrez et al. 2019). Abundant researches described well those little increases in temperature which may stimulate gametogenesis in male and female bivalves which coincided with an elevation of steroid hormones oestradiol, testosterone, and progesterone (Smolarz et al. 2018). In the present study, the effect of temperature was not significant on the estradiol level; however, there was a significant decline in estradiol level when compared to clams treated with 1000 ng/l LNG. This result suggests that an elevated temperature to 24 °C and 28 °C inhibited the effect of the LNG.

The no effect of oestradiol in clams exposed to increasing temperature and levonorgestrel was associated with a high reduction of the number of oocytes and vitellogenin like proteins. In bivalves, oocytes maturation and vitellogenin like proteins biosynthesis have been shown to be modulated by several steroids, most typically estrogens but also progesterone, testosterone and gonadotropins. Evidence of the roles of steroid hormones or other molecules involved in reproduction, development, and maturation in bivalves is scarce and inconclusive. Some authors demonstrated the presence and role of neuropeptides and peptide hormones in physiological functions in some taxa (Ketata et al. 2008; Lafont and Mathieu 2007). For example, receptors for GnRH or GnRH-like peptides have been found in the gonad of the bivalve Crassostrea gigas which are expressed during the gonadal development (Pazos and Mathieu 1999). Other neurohormones (dopamine, noradrenaline, and serotonin) are produced by the nervous ganglia and are playing an important role in reproduction. Lubet and Mathieu 1990 and Mathieu 1994 demonstrated that neurohormones activate both the gamete multiplication and the vitellogenesis, control the mechanisms of energy storage and the spawning process.

Regarding vitellogenin like proteins level responses, our results showed that co-exposure of females of R. decussatus to levonorgestrel and heat stress induced significant decreasing of vitellogenin like proteins levels compared to control. These results are in agreement with those of Cardoso et al. (2019) that demonstrated a decrease in the vitellogenin content of the zebrafish when exposed to a combination of low and high LNG concentrations (10 and 1000 ng/L) and elevated temperatures for 21 days.

In this study, the testosterone level was determined to investigate whether the levonorgestrel and elevated temperature could induce masculinization in female of R. decussatus. A significant reduction of testosterone level was observed in clams treated with high temperatures of 28 °C and with 24 °C associated with LNG. Regarding gonad histological sections, our finding revealed also no masculinization phenomenon was observed. However, many previous studies have demonstrated the masculinization of fish in response to LNG through the AR pathway (Zeilinger et al. 2009; Svensson et al. 2013; Hua et al. 2015; Frankel et al. 2016). Levonorgestrel is derived from testosterone and has a relatively high affinity to the AR (Ellestad et al. 2014).

**Condition index**

Condition index is an ecophysiologically measurement of marine bivalve’s health that reveals changes in the physiological condition of organisms. Condition index characteristics changed with various biotic and abiotic factors such as availability of food, reproduction, physic-chemical characteristics of seawater (dissolved oxygen, salinity, pH, and temperature), and its sensitivity to chemicals (Hassan et al. 2018). Our findings have demonstrated that CI of clams was not significantly altered in response to the increased temperature and/or to levonorgestrel exposure. This could be due to the short-term exposure period of the clam’s treatment. Therefore, CI seems to be less sensitive to eventual physiological changes in clams under the influence of levonorgestrel and heat stress exposure.

**Histological analysis**

The gonads histological examination of R. decussatus revealed a significant alteration by both elevated temperatures of 24 °C and 28 °C and the levonorgestrel (LNG) concentration of 1000 ng/l. The impact of LNG on reproduction has been reported in several studies (Zeilinger et al. 2009; Runnalls et al. 2013; Frankel et al. 2017; Cardoso et al. 2018; Hou et al. 2018), whereas no information is available on bivalves. Our study shows that exposing clams to environmental concentrations of a common progestin LNG disrupted reproduction via a decrease in the number of oocytes within the follicles. The significant reduction in the number of oocytes following exposure to LNG is an important finding because it indicates that oogenesis and fertility in R. decussatus may be at risk from this type of compound. This decrease may be caused by LNG accumulation directly on the gonads or in other peripheral tissues and interfering with endogenous hormones like estradiol, and inhibition gametogenesis. Fick et al. (2010) revealed high LNG bioaccumulation in blood plasma of fish even exceeded the human therapeutic plasma level. Contardo-Jara et al. (2011) also demonstrated that LNG also bioconcentrates in zebra mussels (Dreissena polymorpha) with an interesting phenomenon occurring in the higher exposure concentration (6.24 μg/L). This suggests that LNG could be bioaccumulated during the 28 days with the exposure of clams to 1000
ng/l impaired oogenesis and implying impaired fertility of *R. decussatus*.

The chronic exposure of clams to warming conditions of 24 °C+LNG and 28 °C+LNG caused higher biochemical and physiological effects. The LNG alone caused anti-oxidant defense activation related to catalase (CAT), glutathione -S -transferase (GST), and protein sulfhydryl (PSH) inductions, while the heating diminished most of the specific responses to LNG and was the main factor in the determining of the responses to combine exposures. The estradiol was increased after cam treatment with LNG while it was dramatically reduced after treatment with LNG-28 °C. The chronic heat stress associated to the LNG provoked disruption in clam physiological and biochemical parameters, and this can have negative consequences on the entire organism, species, and ecosystem.

**Conclusions**

The exposure of clams (*R. decussatus*) to levonorgestrel (LNG) alone caused anti-oxidant defense activation related to catalase (CAT), glutathione -S -transferase (GST), and protein sulfhydryl (PSH) inductions, while the heating diminished most of the specific responses to LNG and was the main factor in the determining of the responses to combine exposures. The responses of lysosomal membrane stability, alkaline phosphatase, and NADP+ -dependent isocitrate dehydrogenase detected almost common signs of an adverse effect in all exposures. Testosterone and estradiol responses to LNG were the most particular manifestations depending on the exposure. Therefore, these results showed clearly that chronic warming stress provoked disruption in physiological and biochemical parameters of the clams, which can have implications on the entire organism and community.

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**Author contribution** The authors thank the participants who gave their time to the trial. Jamel Jebali designed the study and revise manuscript. Leila Hmida, Tahar Gharred, and Hamadi Guerbej carried out the treatment experience of clams and supervised the physiological and histological parameters measurements. Asma Mannai and Zied Bouraoui performed the work. All authors contributed to the statistical analysis of the results, the manuscript writing, and approved the final version of the manuscript.

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**Data availability** The data that support the findings of this study are available from the corresponding author upon reasonable request.

**Declarations**

**Ethics approval** All experimental procedures complied with the guidelines regarding the use of laboratory animals of the Bioethical Commission of Scientific Research of the Higher Institute of Biotechnology of Monastir-Tahar Haddad Street, (B.P 74), Monastir, 5000-Tunisia.

**Consent to participate** Not applicable.

**Consent for publication** Not applicable.

**Competing interests** The authors declare no competing interests.

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