Does Thermal Stress Modulate the Biochemical and Physiological Responses of *Ruditapes Decussatus* Exposed to the Progestin Levonorgestrel?

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Abstract

The combined effects of the abiotic factors such as temperature and synthetic progestins on the biochemical and physiological performances of marine clam *Ruditapes decussatus* under sub-chronic conditions are poorly studied. Here, we investigated the effects of levonorgestrel (1000 ng/l) alone or combined with increasing temperature of 20, 24 and 28°C on the biochemical and physiological responses and the gonad histology of the clam (*Ruditapes decussatus*) for 28 days. Our results revealed that, in clams treated with levonorgestrel (LNG) alone, there was an impairment of the defense against oxidative stress related to the inductions of CAT, GST and PSH, a reduction in the lysosomal membrane stability, a physiological disturbance *via* an increase in the level of estradiol, a decrease in the number of gonad oocytes and a reduction in vitellogenin like protein. Furthermore, exposure to heat stress of 20°C, 24°C and 28°C, alone or in combination with LNG, enhanced SOD antioxidant activity which was ineffective in preventing lipoperoxidation and altered clam's physiological processes of reproduction *via* dramatic reduction of gonad oocyte numbers and of vitellogenin like protein reserves. Overall, these findings can be taken as a warning sign of temperature and levonorgestrel progestin effect that can lead to effective changes in the structure and function of target vital organs, such as the gonads of the clam *Ruditapes decussatus*, and this may have implications for the whole organism and populations.

1. Introduction

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 marine animals, including bivalves, for example, 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 Guimarães, 2020; 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 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, 2020). Several studies have reported that levonorgestrel can act as potent endocrine disruptors, 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). 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).

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 *Ruditapes decussatus* is strongly dependent on seawater temperature (Matias et al., 2009, 2016) and the optimum temperature is around 20°C (Delgado and Pérez-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 temperature 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). This toxic situation can be more dramatic when the marine organism is exposed to thermal and chemical stresses; because chemical pollutants can be strongly affected by warming seawater and the sensitivity of organisms to chemicals can be modified 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). 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. 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 "*Ruditapes decussatus*" clam.

The clam (*Ruditapes 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 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 sedentarity, ease of

collection, wide geographic distribution and accumulation of large contaminant capacity from seawater and sediment.

In the present study, the clam *Ruditapes decussatus* was used to investigate the combined chronic effects of the synthetic progestin levonorgestrel and temperature on its physiological and biochemical performances and the histological structure of its gonads. We were particularly interested in the evaluation of responses to oxidative stress, hormonal levels and characteristics of gonadal tissue structures of *R. decussatus* clams exposed to levonorgestrel and heat stress. Our final investigative objective was: Does heat stress modulate the toxicity of levonorgestrel?

2. Materials And Methods

2.1. Clam collection and experimental design

Specimens of *Ruditapes Decussatus*, whose shell length is between 28 to 45 mm, were collected on a reference site of the Tunisian coastal zone. Then they were transported to the laboratory where they are 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 $20 \pm 0.5^\circ\text{C}$, pH 8.2) and fed with microalgae (*Isochrysis galbana*) twice a week (1.10^9 cells/animals). The seawater was renewed every 48 hours.

After acclimatization and to assess the effect of exposure to LNG, under increasing temperature, the clams were randomly divided into six experimental groups of 50 specimens and placed in 20 L glass aquariums with seawater continuously aerated and supplied with microalgae:

Group1: (T20-) ambient temperature of $20^\circ\text{C} \pm 0.5^\circ\text{C}$ and 0 ng / l of levonorgestrel (control condition);

Group 2: (T20+) ambient temperature of $20 \pm 0.5^\circ\text{C}$ and 1000 ng/l levonorgestrel;

Group 3: (T24-) temperature of $24 \pm 0.5^\circ\text{C}$ and 0 ng/l levonorgestrel (warming condition); Group 4: (T24+) temperature of $24^\circ\text{C} \pm 0.5^\circ\text{C}$ and 1000 ng/l levonorgestrel (condition of combined stresses of warming and LNG contamination);

Group 5: (T28-) temperature of $28 \pm 0.5^\circ\text{C}$ and 0 ng/l levonorgestrel (extreme warming condition);

Group 6: (T28+) temperature of $28 \pm 0.5^\circ\text{C}$ and 1000 ng/l levonorgestrel (condition of combined stresses of extreme warming and LNG contamination).

Every 2 days, 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. Levonorgestrel concentration (1000 ng/l) was selected considering the works of Cardoso et al. (2017) and Epeli Manu Loganimoce.

(2106) and the elevated temperatures values chosen based on the range of winter-summer seasonal temperature variation in Tunisia (Kamel et al., 2014).

After the exposure time, the total weight and soft tissues of each clam were measured for condition index (CI) determination.

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 level determinations.

The gonad tissues of clam were carefully removed and 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 determinations.

The digestive gland was dissected and stored at -80°C until biochemical parameters measurements.

2.2. Biochemical parameters determinations

2.2.1. Digestive gland homogenization

Biochemical parameters (superoxide O dismutase (SOD), catalase activity (CAT), glutathione S-transferases activity (GST), lipid peroxidation levels (thiobarbituric acid reactive substances; TBARS), conjugated dienes (CoD) and protein sulfhydryl levels (PSH)) were measured in the digestive gland of control and treated clams. Each parameter was measured in individual clam then 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 x 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. The quantities of proteins present in supernatant were measured spectrophotometrically at 595 nm (Bradford 1976), using bovine serum albumin as the standard.

2.2.2. Oxidative stress biomarkers determinations

Catalase activity (CAT) was determined according to Clairbone (1984) by following the decrease of absorbance at 240 nm due to H₂O₂ decomposition. The reaction mixture (final volume of 1ml) 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 420nm (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 formation of conjugate of glutathione with 1-chloro-2,4-dinitrobenzene (CDNB) at 340 nm for 2 min. The reaction mixture contained cytosolic protein, CDNB (1mM), glutathione reduced form GSH (4mM) and sodium phosphate buffer (100mM, pH 7.5). Results were expressed as $\mu\text{mol GSH-CDNB}$ 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 lipidperoxidation products of polyunsaturated fatty acids and were determined by the method of Recknagel and Ghoshal (1996). 100 μl of tissue homogenate was transferred to a chloroform/methanol mixture (2:1). The whole mixture was vortexed and centrifuged at $2500\times g$ 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/ mg protein.

Protein sulfhydryl (PSH) content was determined, according to Grattagliano et al. (1996). The total sulfhydryl (T-SH) proteins and non-sulfhydryl proteins (NP-SH) were measured. The latter were measured after protein precipitation with trichloroacetic acid (10% TCA). In both analyses, the concentration of TSH and NPSH was determined by its reaction with DTNB (0.01M). The sulfhydryl proteins (P-SH) were determined through the difference between T-SH and NP-SH contents. All measurements were performed at 412 nm. PSH content in the homogenates was expressed as μg per mg of protein.

2.2.3. Alkaline phosphatase activity determination (ALP)

Alkaline phosphatase activity (ALP) was measured by the spectrophotometric method of Nemec and Socha (1988). Enzyme assay included homogenate, 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 spectrophotometer. Results were calculated in terms of nmoles p-nitrophenol /min/mg protein.

2.2.4. Isocitrate dehydrogenase activity determination (ICDH)

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

2.3. Lysosomal membrane stability assay

Lysosomal membrane stability was evaluated by the 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 of five clams from each group was withdrawn from the adductor muscle using a 2 mL syringe with 0.2 mL of physiological 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). 30 µL 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 cells then they 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 dimethylsulfoxide. 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 examined every 15 min under an optical microscope.

2.4. 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 were 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 50 mM potassium phosphate buffer (pH=7.4) and assayed for testosterone and estradiol concentration using commercial RIA kits.

2.5. Determination of vitellogenin like proteins (VTG):

Levels of vitellogenin-like proteins were determined in hemolymph of 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 phosphates 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).

2.6. Condition index determination (CI)

The CI is an interesting indicator of clam general physiological status. The CI of control and treated clams was measured according to Lobel et al. (1991);

CI= (fresh weight of soft tissues/total weight) * 100.

2.7. 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. 5 µm 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.

2.8. Statistical analysis

Statistical analyses were performed using SPSS software. Significant differences between groups were studied using one-way analysis of variance (ANOVA) followed by Duncan's post-hoc test after testing for normality and homogeneity of the data. Differences were considered statistically significant at $p < 0.05$. Different letters a, b, c and d indicated significant differences between groups.

3. Results

3.1. Biochemical responses

Clams treated with levonorgestrel for 28 days at ambient temperature showed significant increased catalase (CAT) (**Fig.1A**) and glutathione S transferase (GST) activities (**Fig.1C**) about 2.2-fold and 1.9-fold when compared to the relative control. The superoxide O 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 and 4-fold, while the isocitrate dehydrogenase activity (ICDH) (**Fig.1H**) was reduced 2-fold when compared to the control.

The clams exposed only to various temperature degrees (20°C, 24°C and 28°C) exhibited alteration of biochemical parameters marked by high reduction of CAT (**Fig.1A**) and GST (**Fig.1C**) activities. In contrast, SOD activity was enhanced in temperature increase dependant manner. The increase was about 1.37-fold at 24°C and 1.84-fold at 28°C when compared to the relative control maintained at 20°C. The PSH level was increased 6.49-fold in clams exposed to 24°C and 6.68-fold at 28°C. The thiobarbituric acid reactive substances (TBARS, **Fig.1D**) and conjugated dienes levels (**Fig.1E**) were not significantly changed except in clams exposed to 24°C where the TBARS increased about 1.85-fold. The ICDH activity was significantly inhibited in clams exposed to 24°C as opposed to clams exposed to 28°C (**Fig.1H**).

Clams exposed to LNG +24°C and to LNG + 28°C showed decrease 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 while ICDH activity was reduced in treated clams with the exposure to LNG +24°C and LNG + 28°C comparing with the control. The thiobarbituric acid reactive substances (TBARS, **Fig.1D**) and conjugated dienes (**Fig.1E**) levels were significantly enhanced in treated clams with LNG +24°C and LNG + 28°C. A more remarkable increase in conjugated dienes levels was observed in clams exposed to LNG + 28°C when compared to the clams exposed only to 28°C where the increase was about 1.53-fold.

3.2. Lysosomal membrane stability assessment

Lysosomal membrane integrity of haemocytes was evaluated in clams treated with levonorgestrel and 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 of the NRRT about 18.42% was observed in clams exposed to 1000 ng/l LNG at ambient temperature of 20°C when compared to the relative control (**Fig.1I**). Clams exposed to the increasing temperatures of 24°C and 28°C showed a higher decrease of NRRT about 68.42% and 81.57% when compared to the control group. High reductions of NRRT were observed in clams coexposed to the levonorgestrel and heat stress (LNG+ 24°C and LNG+ 28°C (**Fig.1I**)).

3.3. Physiological responses

3.3.1. Gonad steroids concentrations

Our results indicated that females treated with 1000 ng/l LNG exhibited a significant high gonad estradiol level (2.23-fold) (**Fig. 2B**) and non-significant low testosterone level (**Fig.2A**). The exposure to the heat stress (24 and 28°C) induced a decrease of estradiol and testosterone levels with significant diminution of testosterone at 28°C. Clams exposed to LNG +24°C exhibited non-significant low estradiol level while the testosterone level was significantly low. A non-significant decrease of estradiol and testosterone levels was recorded in clams co-exposed to 28°C+ LNG compared with control group.

3.3.2. Determination of vitellogenin like proteins (VTG): ALP assay

Females exposed to the temperatures only (24 and 28°C) showed a decrease of vitellogenin like proteins levels when compared with control clams (**Fig.2C**). 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 (**Fig.2C**).

3.4. Condition index (CI)

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

3.5. Histological analysis

Control female gonads were characterized by normal gonadal tubules with many free mature spherical oocytes occupy the lumen (**Fig.3.A**). Exposure to 1000 ng/l LNG inhibited gonadal development and significantly decreased the number of oocytes per follicle than those of the control (**Fig.3.B**). The decrease in the number of oocytes per follicle area was approximately (58%) compared to the control group. The increase of temperature (24°C) had a positive effect on maturation of female clams. Results depicted in **Fig.3.C** 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 completely absence of oocytes (**Fig.3.D**). In exposed females to the combined LNG+ 24°C, atrophied follicles and the central lumen was nearly empty, only a few residual oocytes were found at the periphery of the follicles (**Fig.3.E**). Similarly, coexposed females to the increasing temperature and levonorgestrel (LNG+ 28°C) showed empty (0 oocytes per follicle) and atrophied of ovarian follicles. **Fig.3.F** showed also an increase in the inflammatory response characterized by a focal or diffuse haemocytic infiltration and formation of haemocytic aggregates (granulocytomas) in the connective tissue.

4. Discussion

4.1. Biochemical responses

Synthetic progestins in marine ecosystem have adverse effects on the marine sedentary organisms like bivalves even at very low concentration of ng/l. Abundant studies available on the potential endocrine disruption effects and reproduction alteration of the synthetic hormones and the physiopathology mechanisms in non-target organisms were explored. However, a very few data are focused on the combined effect of the temperature and synthetic progestins such as levonorgestrel on the biochemical of marine bivalves like clams. In the present study, we investigated the effects of various degrees of temperature of 20, 24 and 28°C combined to 1000 ng/l levonorgestrel on the physiological and biochemical performances of clams (*Ruditapes decussatus*) for 28 days. Therefore, our evaluation may be the first treating this approach.

The exposure to the 1000 ng/l LNG exhibited marked oxidative stress generation and we noted a significant increase of catalase (CAT), glutathione-S-transferase (GST) and protein sulfhydryl (PSH) levels. The enhanced antioxidative defense inactivates lipidperoxidation products, such as lipid hydroperoxides and their derivatives as well as reactive oxygen species (ROS) by conjugation to glutathione (Doyen et al., 2005). In this study, higher GST and CAT activities in contaminated clams by LNG were accompanied by lower conjugated dienes (CoD) and thiobarbituric acid reactive substances (TBARS) levels which may indicate the capacity of organisms to eliminate the excess of ROS and prevent membranes 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, antioxidant defense and protein damages (Contardo-Jara et al., 2011).

Temperature is one of the important environmental factors that controls biochemical, physiological, 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 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 ability to cope with thermal stress (Nardi et al., 2018 ; Costa et al., 2020). Our results revealed that the combined effect of levonorgestrel and 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 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 antioxidant and detoxifying enzymes.

Others 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 molecule permeability, protein synthesis, growth and cell differentiation, gonadal maturation and steroidogenesis (Ram and Sathayanesan, 1985). Although, this enzyme has been considered as useful biomarker because of its sensitivity to 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 (Ranilalitha et al., 2014; Nandurkar., 2017), very few 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 comparing with control. Hansen et al. (1992) explained that increases in ALP activity resulting in changes of metabolic activity allowing adaptation or organism to submitted stress.

NADP-dependent isocitrate dehydrogenase (ICDH) is an essential enzyme that catalyzes oxidative decarboxylation of isocitrate to α -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 antioxidant 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 consequence a decrease in its capability to deal with high oxygen reactive species generation.

4.2. 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 provoke diminution of its integrity (Shaw et al., 2019; Parisi et al., 2021). Therefore, its usefulness in several biomonitoring exercises explained by its none destructive 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 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.

4.3. Physiological responses

Endogenous gonads hormones including 17 β - 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). One of the primary functions of endogenous gonads hormones are 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 oestradiol 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 we hypothesis that LNG and its metabolites 5 α -LNG, 3 α ,5 α -LNG and 3 β ,5 α -LNG could mimic estradiol and bind to its receptor and inhibit the gonad development, oocyte maturation and vitellogenin like proteins synthesis. This hypothesis 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 glycopospho-lipo-proteins synthesized by mature gonads of bivalves and stored in developing oocytes. It has been shown that vitellogenin like proteins under control of 17 β - oestradiol interacting with the estrogen receptor (Osada et al.,2003). Our results indicate that clams exposed to LNG only for 28 days caused lower decreasing of VTG level in hemolymph comparing with control. These results are in part corroborated by the observations of Zucchi et al. (2014) which detected a downregulation of vitellogenin like proteins in the zebrafish liver when exposed to the synthetic progestin 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 is usually considered a good biomarker to

detect estrogenic contamination and be also elicited by aromatized androgens, *in-vitro* (Kim et al., 2003) and *in-vivo* (Madureira et al., 2018). In our case, this decrease may be also explained by binding of LNG to VTG receptor causing blocked estrogen binding to this receptor or it can be associated to the androgenic activity of LNG (Hua et al., 2015). Besides, it can be explained by LNG interfere with the release of nervous system releasing neurohormones to effectively alter the production of VTG (Zucchi et al., 2014; Zhao et al., 2015b).

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 coincided with an elevated 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 is a significant decline in estradiol level when compared to clams treated with 1000 ng/l LNG. This result suggests that elevated temperature to 24°C and 28°C inhibited the effect of the LNG.

The non-significant reduction of oestradiol in clams exposed to increasing temperature and levonorgestrel was associated with a high reduction of number of oocytes and vitellogenin like proteins level. 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 the 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 *Ruditapes decussatus* to levonorgestrel and heat stress induced significant decreasing of vitellogenin like proteins levels comparing with control. These results are in agreement with those of Cardoso et al., (2019) that demonstrated decreasing in the vitellogenin content of the zebrafish when exposed to the combined of low and high LNG concentrations (10 and 1000 ng/L) and elevated temperatures for 21 days.

Testosterone is a steroid hormone associated with male bivalve gonads development and spermatozooids maturation. In this study, testosterone level was determined to investigate whether the levonorgestrel and elevated temperature could induce masculinization in female of *Ruditapes 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).

4.4. Condition index (CI)

Condition index is an ecophysiological 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., 2108). Our findings have demonstrated that CI of clams did 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.

4.5. Histological analysis

The gonads histological examination of *Ruditapes decussatus* revealed a significant alteration by both elevated temperatures of 24°C and 28°C and the levonorgestrel (LNG) concentration of 1000ng/l. The impact of LNG on the 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 exposure 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 1000ng/l, impaired oogenesis and implying impaired fertility of *Ruditapes decussatus*.

5. Conclusions

This study proves that exposure of clams (*Ruditapes decussatus*) to levonorgestrel (LNG) alone caused oxidative stress defense alterations related to catalase (CAT), glutathione -S -transferase (GST) and protein sulfhydryl (PSH) inductions, reduction of lysosomal membrane stability and physiological disruption *via* estradiol level diminution, gonads oocytes number reduction, and vitellogenin like proteins

diminution. Furthermore, our results showed clearly that exposure to heat stress of 20, 24 and 28°C alone or in combination with levonorgestrel (LNG) enhanced superoxide dismutase (SOD) antioxidant activity that was ineffective to avoid lipoperoxidation increase, dramatic alteration of clam physiology processes and reproduction success *via* high reductions of gonads oocytes number and vitellogenin like proteins reserves. Therefore, these results can be considered as a warning sign that combined factors such as temperature and levonorgestrel (LNG) can lead to effective changes on the structure and functioning of target vital organs, like the gonads of clams, and this can have implications on the entire organism and community.

Declarations

Author contributions

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.

Data availability statement

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

Declaration of Competing Interest

The authors confirm that there are no conflicts of interest in this work.

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Ethical 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

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Consent to participate

Not applicable

Consent to Publish

Not applicable

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Figures

Figure 1

Biochemical parameters responses and lysosomal membrane stability of the clam (*Ruditapes decussatus*) exposed to the increasing temperature of 20, 24, and 28°C associated to the 1000ng/l progesterin levonorgestrel for 28 days. (A): Digestive gland catalase activity (DG-CAT); (B): Digestive gland Superoxide dismutase activity (DG-SOD); (C): Digestive gland Glutathione-S-transferase activity (DG-GST); (D): Digestive gland Thiobarbituric acid reactive substance level (DG-TBARS); (E): Digestive gland

Conjugated dienes (DG-CoD); (F): Digestive gland Protein sulfhydryl (DG-PSH); (G): Digestive gland Alkaline phosphatase (DG-ALP); (H): Digestive gland Isocitrate dehydrogenase (ICDH) and (I): Lysosomal membrane stability. Data represented as means $\pm$ SD. Different letters a, b, c, and d indicated significant difference between clam groups.

Figure 2

Physiological parameters responses of the clam (*Ruditapes decussatus*) exposed to the increasing temperature of 20, 24, and 28°C associated to the 1000ng/l progesterin levonorgestrel for 28 days. (A): Testosterone level; (B): Estradiol level; (C): Vitellogenin like proteins level (VGT); (D): Condition index (CI); (E): Number of oocytes per visual field. Data represented as means $\pm$ SD. Different letters a, b, c, and d indicated significant difference between clam groups.

Figure 3

Histological sections of female clam gonad (*Ruditapes decussatus*) exposed to the increasing temperature of 20, 24, and 28°C associated to the 1000ng/l progestin levonorgestrel (LNG) for 28 days. Control clams (T20-) exhibited normal ovarian follicles (OF) containing maturing oocytes (OC) (A). Atrophied follicles, reduction in the oocytes number and only residual oocytes at the periphery of female gonads of clams exposed to LNG+20°C (T20+) (B) and LNG+24°C (T24+) (D). Gonad of clams exposed to the elevated temperature of 24°C (T24) showing follicles filled with mature oocytes (C). Empty gonad of clams treated with elevated temperature of 28°C after total oocytes emission (T 28-) (E). Highly

affected gonads of clams exposed to 28°C and LNG (T28+) with empty and atrophied of follicles and presence of haemocytic infiltration in connective tissue (ct) indicating gonad inflammation of (F).

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