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Acute exposure of cadmium on *Donax trunculus* Linnaeus, 1758 (Mollusca Bivalvia) during the vitellogenesis process: histological and biochemical aspects

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ABSTRACT This work aims to evaluate the impact of cadmium on the vitellogenesis of *Donax trunculus* Linnaeus, 1758 an edible Mollusk species, by carrying out a histological study in which the morphometric parameters of the oocytes were measured, and by using vitellogenin (Vtg) and vitellin (Vn) as biomarkers of reprotoxicity. Clams were collected from a clean site (El-Battah) during the period of morphological maturity and reared under laboratory conditions. Cadmium chloride was added to the rearing water at two sublethal concentrations (LC10 and LC25-96h) previously determined. Two-way ANOVA revealed significant effects of Cd concentrations and exposure time on all studied parameters. The data obtained suggest that this metal can act as endocrine-disrupting chemicals in *Donax trunculus*.

KEY WORDS Cadmium; oocyte; vitellin; vitellogenin; vitellogenesis.

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INTRODUCTION

Heavy Metals, as elements, are not biodegradable; although their chemical state may be altered and form compounds of varying toxicity to aquatic organisms, their toxicity varies according to environmental conditions (George et al., 2013). Over the last decades, contamination by metallic compounds has become a subject of concern worldwide; indeed, marine pollution related to heavy metals raises many concerns for aquatic populations and humans (Singh et al., 2021; Yap et al., 2021).

The Algerian coastline, which extends for more than 1200 km, is considered a coastal ecosystem of great ecological importance. They are home to various animal and plant species. Pollution of these coastal areas by various chemical substances can have serious consequences on the ecological balance (Houma et al., 2011). Previous works have shown that the gulf of Annaba is affected by various heavy metals (Abdennour et al., 2000; Rabei et al., 2018 ; Amira et al., 2018). Indeed, cadmium was particularly detected in sediments and tissues of an abundant edible species *Donax trunculus* Linnaeus, 1758 (Mollusca Bivalvia Donacidae), in the bay of Annaba (Beldi et al., 2006; Drif et al., 2010; Amira et al., 2018). This species has been used as a bioindicator of marine coastal waters' quality due to their sedentary and sessile lifestyles. As filter feeders, their tissues tend to bio-accumulate many contaminants at higher levels (Hamdani et al., 2020; Boukari et al., 2021).

For many years, cadmium (Cd) has been considered one of the most dangerous toxic heavy metals (Järup, 2003; Xie et al., 2014) because of its intrinsic ionic similarity to calcium. Indeed, marine bivalves can accidentally take up cadmium and pass it into their cells through calcium channels (Vercauteren & Blust, 1999; Shi et al., 2018). It can cause a series of biochemical and physiological dysfunctions both in humans and laboratory ani-

mals (Santovito et al., 2015; Senthamilselvan et al., 2016 ; Silva et al., 2017), such as the depletion of energy reserves (Merad & Soltani, 2017), oxygen consumption increase (Chandurvelan et al., 2017) and damage to DNA (Michel & Vincent-Hubert, 2015). Likewise, cadmium can have an endocrine-disrupting power. The correlations between the involvement of cadmium in the dysfunction of energy status and endocrine disruption may interfere with the reproduction of bivalves (Ketata et al., 2007; Baudrimont et al., 2019).

Histological study of gonad through morphometric measurements of oocytes can be considered good biomarkers for evaluating environmental endocrine disruption; while vitellogenin and vitellin are widely accepted as biomarkers to assess estrogenic disruption in aquatic environments (Blaise et al., 2003; Matozzo et al., 2008). In benthic bivalve molluscs, the vitellogenin (Vtg) is a glycolipophosphoprotein synthesized by the digestive gland and secreted in the hemolymph. It is transported to the ovaries or accumulates in the oocytes in growth (Vitellogenesis) by a connection with specific receptors associated with endocytose vesicles; this protein is the precursor of vitellins reserves (Vn) (Wahli et al., 1981; Denslow et al., 1999; Jubeaux, 2015). Besides, vitellin is a glycolipoprotein of egg necessary to develop the future embryo (Robinson, 2002).

The synthesis of the precursor of Vn was shown as being controlled by the oestrogens in fresh water and sea bivalves (Blaise et al., 1999; Blaise et al., 2003; Quinn et al., 2004; Marin and Matozzo, 2004). This study aims to assess the sub-lethal effects of cadmium (LC_{10} and LC_{25}-96h) on morphometric measurements of oocytes of *D. trunculus* using histological analysis and on the level of vitellogenin and vitellin in hemolymph's and gonad's of *D. trunculus,* respectively.

MATERIAL AND METHODS

Samples collection and treatment

Specimens of adult *D. trunculus* were collected from El Battah beach (36° 50' N - 7° 50' E) in March 2018, where the majority of individuals were then in the stage of morphological ripe (Hamdani et al., 2020). El-Battah beach was chosen as a sampling site because of its remoteness from anthropogenic activity, its important hyrodynamic exposure, and the species' abundance (Fig. 1) (Rabei et al., 2018; Boukari et al., 2021). Animals were transported in cold boxes to the laboratory, and *D.trunculus* females were separated by macroscopic inspection according to the gonads colour: dark blue. Clams were acclimatized for 48h before exposure to cadmium chloride (Belabed & Soltani, 2013). The water was constantly aerated during all the experiments, and a 12h light/dark cycle was maintained**.** The physico-chemical parameters of seawater were as follows: temperature: 16.65 ± 0.49 °C; salinity: 33.50 ± 0.87 g/L; pH: 8.09 ± 0.037; dissolved oxygen: 7.91 ± 0.37 mg/L. *D. trunculus* were fed daily with a commercial food mixture (Marine Invertebrate Diet Carolina Ltd., NC, USA) and were exposed to cadmium chloride (CdCl2), according to 96h-LC_{10} (0.94mg /L) and 96h-LC_{25} (1.60mg /L) as previously determined by Merad & Soltani (2015). The acute toxicity was measured for 96h.

Figure 1. Localisation of sampling site: El Battah beach

Histological procedures

The histological procedure was performed according to Gabe (1968). In brief, gonads were fixed in alcoholic Bouin's solution for 24–48 h. Then, tissues were carried out in alcohol baths of increasing concentration, as follows: 70%, 80%, 90% and 95%. Tissues were embedded in paraffin wax at 60 ˚C. After setting in a block, in plastic cassettes, the microtome cuts the blocks to a thickness of 5 µm. Sections were stained with hematoxylin and eosin, mounted in Canada balsam. Slides were blindly examined usinga Leica microscope (DM500) equipped with a Leica camera (ICC50 HD). The morphometric parameters, such as length (L) and width (l), were measured with a rule associated

with a Leica camera and were expressed in µm. The volume was calculated according to the following formula (Lumbreas et al., 1991) and was expressed in mm^3as follows: $V= 4\pi/ 3(L/2) (l/2)$.

Vitellogenin and vitellin determination

The Vtg and Vn levels were made on hemolymph and gonads of *D. trunculus,* respectively. The extraction was performed using a Tris buffer (0.5 M; pH 7.4) following the procedure of Fabre et al. (1990). Samples were homogenized by ultrasound and then centrifuged (5,000 g for 10 min). Three distinct layers were separated in hemolymphand gonads samples, and the intermediate layer containing the Vtg and Vn respectively was removed and stored at 20°C until analysis. The levelsofvitellogenin and vitellin were made according to Bradford (1976) using Coomassie brilliant blue G-250 (CBB) as a reagent and bovine serum albumin (BSA) as standard (Sigma). The absorbance was read at a wavelength of 595 nm.

Data analysis

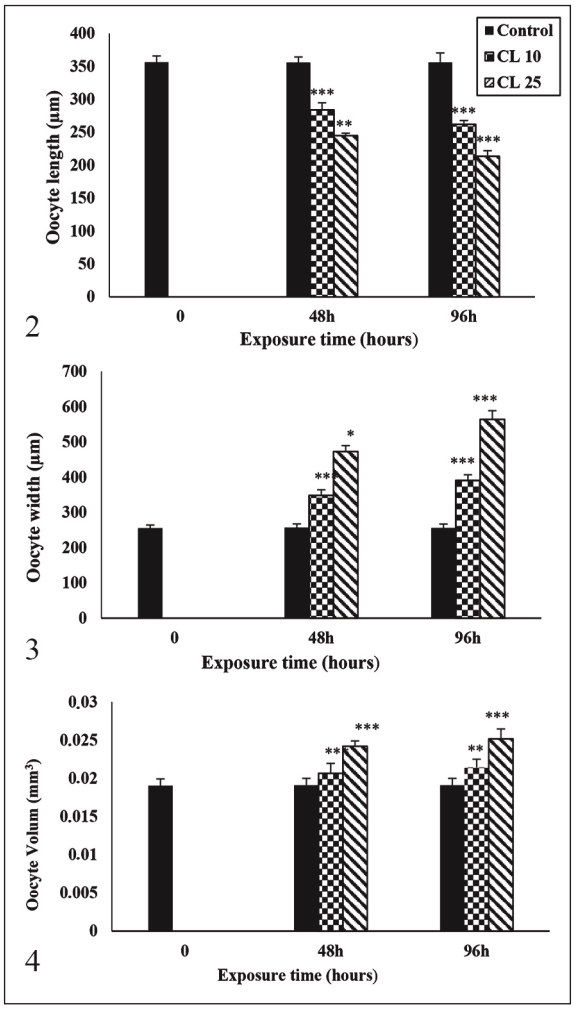
Statistical analyses were performed using Prism version 7 for Windows (GraphPad software, La Jolla, CA, USA, www.Graphpad.com). Data are expressed as mean ± standard deviation (mean ± SD) with a statistical significance level of $p < 0.05$. A two-way analysis of variance (ANOVA), followed by Tukey's post-hoc test, was used to evaluate differences between the control and treated series. Pearson correlation-test determined the correlations between all parameters studied.

RESULTS

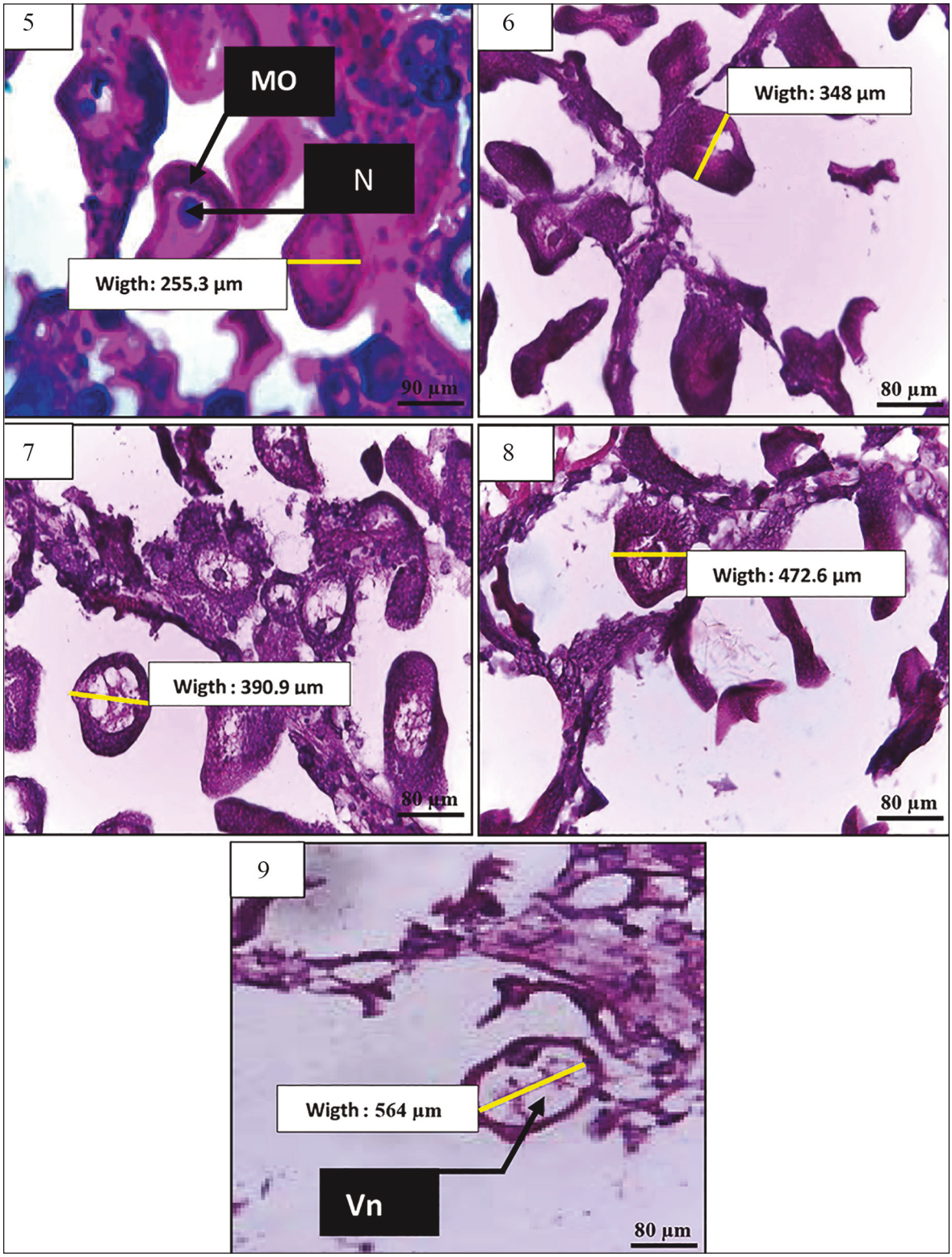
Sublethal effects of cadmium on morphometric measurements of oocytes

The impact of sub-lethal concentration of Cd on the morphometric measurements of oocytes was represented in Figs. 2–4 and Figs. 5–9. There was no significant difference between the morphometric measurements of oocytes recorded during the experimental period in the control series. However, the treated series presented a significant difference compared to the control series during the experimental period. As shown in Fig. 2, the treated series indicated a significant decrease in oocyte length compared to the control series. Furthermore, oocyte length differed significantly between the control and LC_{10-} treated series at 48h and 96h ($p = 0.001$), and between the control and LC_{25-} treated series at 48h ($p = 0.001$) and 96h ($p = 0.000$). A two-way ANOVA revealed significant effect of concentrations ($F_{2.36} = 311.9$; $p < 0.0001$) and exposure time ($F_{2.36} = 293$; $p< 0.001$) and a significant effect of time x treatment interaction ($F_{4.36}= 81.71$; $p = 0.0001$).

Figures 3 and 4 show that a significant increase of oocyte width and volume as compared to the control series. Indeed, the oocyte width differed



Figures 2–4. Effects of cadmium on morphometric measurements of oocytes in *D.trunculus.* Fig. 2: effect of Cd on the length of oocytes. Fig. 3: effect of Cd on the width of oocytes. Fig. 4: effect of Cd on the volume of oocyte) (mean ± SD; n = 5). Asterisks above treated series indicated significant difference with controls of the same time (*: significant difference at $p < 0.05$; **: significant difference at $p < 0.01$; ***: significant difference at $p < 0.001$.



Figures 5–9. Histological sections of the ovaries of *Donax trunculus* in sexual maturity (Gx40). Fig. 5: 0j; Control. Fig. 6: CL10(48h). Fig. 7: CL10(96h). Fig. 8: CL25(48h). Fig. 9: CL25(96h). N: Nucleus; MO: Mature oocyte; Vn: vitellin.

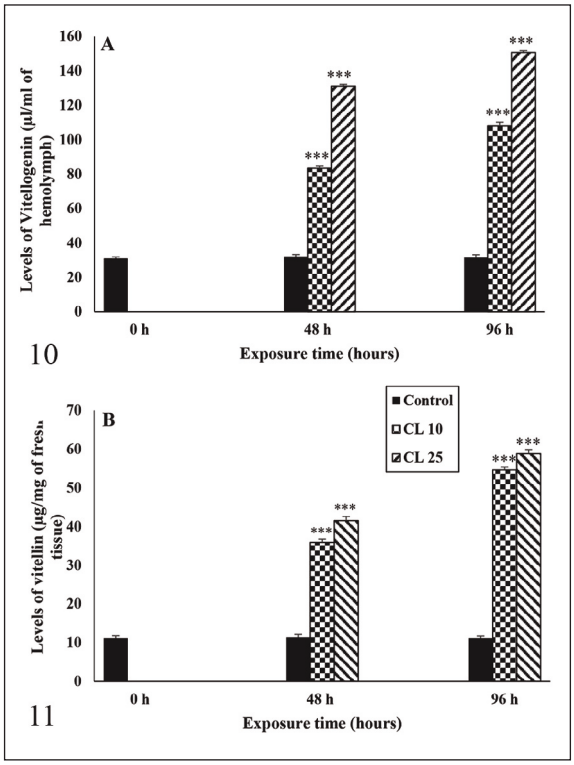
significantly between the control series and the LC_{10}-treated series at 48h and 96h ($p = 0.001$), and between the control series and the LC_{25}-treated series at 48h ($p = 0.001$) and 96h ($p = 0.000$). A two-way ANOVA recorded a significant effect of concentrations ($F_{2.36} = 216.1$; $p < 0.0001$) and exposure time ($F_{2.36} = 161.1$; $p < 0.001$) and a significant effect of time x treatment interaction ($F_{4.36} = 59.04$; $p = 0.0001$). In addition, the oocyte volume diverged significantly between the control and LC_{10}-treated series 48h ($p=0.001$) and 96h ($p = 0.001$), and between the control and LC_{25}-treated series 48h and 96h ($p = 0.000$). This result was confirmed by two-way ANOVA where significant effects of concentrations ($F_{2.36} = 47.41$; $p < 0.0001$) and exposure time ($F_{2.36} = 30.4$; $p < 0.001$) and time x treatment interaction ($F_{4.36} = 12.14$; $p = 0.0001$) were observed.

Sublethal effects of cadmium on vitellogenin and vitellin levels

The sublethal effects of Cd on the levels of Vtg and Vn varied as function time and concentration. Like morphometric parameters on the control series, no differences between the values of Vtg and Vn levels were noted during the experimental period. Nevertheless, the treated series presented a significant increase in Vtg and Vn levels compared to the control series (Figs. 10, 11). Also, Vtg levels were significantly different between control and LC_{10}-treated series 48h and 96h ($p = 0.001$), and between control and LC_{25}-treated series 48h ($p = 0.001$) and 96h ($p = 0.000$) (Fig. 10). A two way ANOVA-showed a significant effects of concentrations ($F_{2.36} = 5874$; $p < 0.0001$) and exposure time ($F_{2.36} = 5450$; $p < 0.001$) and significant effect of time x treatment interaction ($F_{4.36} = 15.33$; $p = 0.001$). Likewise, Vn levels were found to differ significantly between the control and LC_{10}-treated series at 48h and 96h ($p = 0.0001$) and between the control and LC_{25}-treated series at 48h ($p = 0.0001$) and 96h (0.0001) (Fig. 11). A two-way ANOVA revealed a significant effect of concentrations ($F_{2.36} = 3271$; $p < 0.001$) and exposure time ($F_{2.36} = 3758$; $p < 0.001$) and a significant time x treatment interaction ($F_{4.36} = 949.4$; $p < 0.001$).

Correlation tests

The Pearson correlation tests between all parameters studies in *D. trunculus* are displayed in Table 1. The results showed a highly significant ($p= 0.001$) negative correlation between levels of Vtg and length of the oocyte, levels of Vn and length of the oocyte, length and width of the oocyte, length and volume of the oocyte. A highly significant ($p=0,001$) positive correlation was revealed between levels of Vtg and Vn, Vn and width of the oocyte, Vn and volume of the oocyte,Vtg and width of the oocyte, Vtg and volume of the oocyte, width and volume of the oocyte.



Figures 10, 11. Sublethal effect of cadmium on vitellogenin and vitellin levels in *D. trunculus*. Fig. 10: effect of Cd on vitellogenin levels (µl/ml of hemolymph). Fig. 11: effect of Cd on vitellin levels (µg/mg of fresh tissue) (m ± SD; n = 5). Asterisks above treated series indicated significant difference with controls of the same time (*: significant difference at $p < 0.05$; **: significant difference at $p < 0.01$; ***: significant difference at $p < 0.001$.

DISCUSSION

Several reproductive processes can be targeted by contaminants that will alter both hormonal actions and reproductive physiology (Gagné et al., 2002; Baudrimont et al., 2020). Indeed, the structural and functional disruptions caused by pollutants and their metabolites lead to physiological and be-

Parameters	R	P
Length – width	-0.956	0.001
Length – volume	-0.834	0.001
Width – volume	0.940	0.001
Vitellogenin – length	-0.985	0.001
Vitellogenin – width	0.977	0.001
Vitelogenin – volume	0.896	0.001
Vitellin – vitellogenin	0.959	0.001
Vitellin – length	-0.960	0.001
Vitellin – width	0.910	0.001
Vitellin – volume	0.803	0.001

Table 1. Pearson correlation tests between all parameters studied in *D. trunculus* (R = coefficient of correlation; P = significance level).

havioural performance changes, including steroidogenesis and vitellogenesis (Benelli et al., 2001; Arab et al., 2004; Ortiz-Zarragoitia & Cajaraville, 2005; Zheng et al., 2010; Paschoalini et al., 2019).

In the present study, the results indicate a significant increase in oocyte width and volume, and a significant decrease of the length of oocytes in *D. trunculus* treated series compared to the controls series at two sublets concentration. This could be explained by the increase of Vn levels in oocytes, as reported in oysters (Fabiola et al., 2009), which shows that the increase in Vtg concentrations is synchronized with oocyte surface area and diameter. In addition, several studies on bivalves and invertebrate species have reported a positive correlation between Vtg, Vn concentrations and oocyte diameter (Vazquez-Boucard 1990; Quinitio & Millamena 1992; Li et al., 1998).

Concerning the Vtg and Vn levels, it has been reported that the estrogens factor regulates the synthesis of Vtg in marine bivalves (Quinn et al., 2004). The results show a significant increase in Vtg and Vn levels in *D. trunculus* treated series compared to controls series at two test concentrations and during the exposure periods. These results confirm our histological studies and could explain that some endocrine disruptors such as metals can bind to estrogen receptors and induce agonistic responses to estrogen. These xenoestrogens have been called metalloestrogens (Quinn et al., 2004; Darbre, 2006; Paschoalini et al., 2019). These results are in good agreement with previous studies on bivalves such as *Mytilus edulis* following in vivo injection of nonylphenol (Blaise et al., 1999) and after exposure to acute toxicity of copper (Cu) (Zorita et al., 2006), and *Elliptio complanata* after in vivo injection of nonylphenol (Gagné et al., 2001). *E. complanata* transplanted in highly contaminated municipal effluents (Blaise et al., 2003). *Dreissena polymorpha* after exposure to effluents, suggesting an endocrine disruption (Quinn et al., 2004), *Argopecten gibbus* after a semisubmerged municipal dump (Quinn et al., 2005). Similar results have been observed in other marine invertebrates such as *Asteria rubens* (Schoenmakers et al., 1981) and the crustacean *Balanus amphitrite* (Billingurst et al., 2000).

CONCLUSIONS

The present study confirms the metalloestrogenic character of Cd for *Donax trunculus* by an endocrine disruption reflected by an increase of morphometric parameters of oocytes, exception the length and the levels of vitellogenin and vitellin.

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