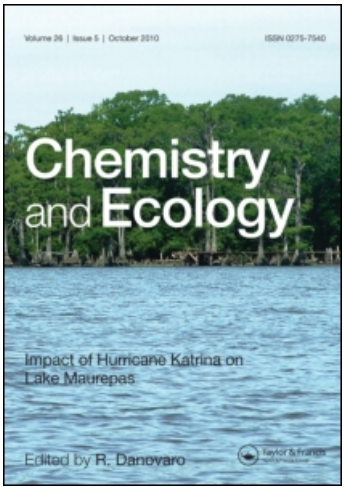

Chemistry and Ecology
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713455114

Can estrogenic compounds enhance the activity of cathepsin D and cathepsin L in the mussel, *Mytilus galloprovincialis*?

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To cite this Article Margiotta-Casaluci, Luigi and Carnevali, Oliana(2009) 'Can estrogenic compounds enhance the activity of cathepsin D and cathepsin L in the mussel, *Mytilus galloprovincialis*?', Chemistry and Ecology, 25: 1, 49 — 60
To link to this Article: DOI: 10.1080/02757540802609392
URL: http://dx.doi.org/10.1080/02757540802609392

Chemistry and Ecology
Vol. 25, No. 1, February 2009, 49–60

Taylor & Francis
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RESEARCH ARTICLE

Can estrogenic compounds enhance the activity of cathepsin D and cathepsin L in the mussel, *Mytilus galloprovincialis*?

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(*Received 1 September 2008; final version received 7 November 2008*)

The aim of this study was to obtain new information on two lysosomal enzymes, cathepsin D (Cat D) and cathepsin L (Cat L) in the Mediterranean mussel, *Mytilus galloprovincialis*, and to test in a preliminary way if Cat D and Cat L enzymatic activity can be enhanced in response to estrogenic compounds, such as nonylphenol (NP) and estradiol (E_2). In addition to assay optimisation, a number of experiments were performed in order to assess Cat D natural seasonality, the effect of captivity on Cat D and Cat L activity, and the response to estrogenic compounds. Exposure experiments were conducted under static conditions; once exposed to NP (300 μg/l) and E_2 (1000 μg/l) on day one, mussels were sampled after 7 and 14 days. The main registered effect was a significant increase of Cat D activity in the digestive gland, caused by both NP and E_2. The results obtained provide preliminary information on Cat D and Cat L in the *Mytilus galloprovincialis*; furthermore the Cat D activity enhancement, caused by estrogenic compounds, provides a starting point for further studies designed to evaluate if the Cat D assay in mussels may supply indications on the presence of estrogenic compounds in the environment.

Keywords: endocrine disruptors; nonylphenol; *Mytilus galloprovincialis*; cathepsin D; cathepsin L

1. Introduction

It is well-known that the normal functioning of the endocrine system can be disrupted by a number of anthropogenic and naturally occurring chemicals, thereby affecting physiological processes under hormonal control [1]. Chemical compounds mimicking estrogen effects, such as alkylphenols, alkylphenol ethoxylates, PCBs, dioxins, and various pesticides are considered to be endocrine disruptors (EDs) and have been found in hazardous concentrations in estuarine, freshwater and marine environments [2]. The adverse effects of EDs have been observed in many organisms [3–7]; however, although ED effects and mechanisms are well-known in vertebrate species, very little information is available when considering invertebrates, despite the fact that

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ISSN 0275-7540 print/ISSN 1029-0370 online

DOI: 10.1080/02757540802609392
http://www.informaworld.com

they constitute ca. 95% of all animal species and are key components in all ecosystems [8–11]. Thus, in this study we used the Mediterranean mussel, *Mytilus galloprovincialis*, as an experimental model, because of its wide use in monitoring environmental pollution programs (e.g. 'Mussel Watch') [12], its important role in the human diet, and consequently its potential role as a route for exposure to EDs in humans.

Vitellogenin (Vg) is a classic biomarker of exposure to estrogen-mimicking chemicals; however its use in molluscs is still controversial. In bivalves, information on vitellogenesis is still limited; it has been hypothesised that vitellogenesis occurs in the gonad where the oocytes are believed to produce yolk proteins autosynthetically [13–15] through a process induced and regulated by estrogens [16]. On the other hand, Eckelbarger and Davis (1996) argued that vitellogenesis can occur also through the heterosynthetic process, in which extraovarian precursors can be incorporated into oocytes via receptor-mediated endocytosis from the hemocoel [17]. However, using one-dimensional and two dimensional PAGE, Riffeser and Hock (2002), did not find any change of the protein pattern in the hemolymph of the marine mussel *Mytilus* or the freshwater mussel *Anodonta* in response to E_2, suggesting that the presence of Vg-like proteins in the hemolymph may originate from gonadic tissue breakdown products [18].

Levels of Vg-like proteins in bivalves are usually determined using an indirect method, the alkali-labile phosphate assay [19–23]. Although this method has proved to be highly correlated with other assays for Vg [19,24], it cannot provide a quantitative measurement of Vg-like protein concentration, and sometimes is not able to provide conclusive results [25].

At a molecular level, Vg induction by estrogens seems unclear; some authors reported that in *Mytilus edulis* gonadal tissue, exposure to 200 ng/l of E_2 did not produce any significant change in the expression of the Vg gene over the 10-day period examined, but an extremely high individual variability was observed and there were no recorded differences between males and females with the same treatment [26].

It is clear that looking for new biomarkers of exposure to estrogen-mimicking chemicals and other EDs in molluscs and other invertebrates is a crucial issue for the future, in order to extend the control of this hazardous class of pollutants to new species. In this study, we focused our attention on two lysosomal enzymes: cathepsin D (Cat D) and cathepsin L (Cat L).

Cat D is a member of the aspartic protease family and is involved in various physiological pathways, including intracellular proteolysis, enzyme and hormone processing, secretion and activation [27,28]. In oviparous vertebrates, it has been demonstrated that Cat D plays a key role in reproduction, being involved in yolk formation in the ovary during vitellogenesis [29,30] and in yolk mobilisation during early embryogenesis [31,32]. Estrogens can stimulate Cat D gene expression, after binding the estrogen receptor, acting on the estradiol responsive element (ERE) at the promoter region of the gene [33]. Carnevali and Maradonna (2003) provided the first experimental evidence documenting that the Cat D gene and its enzymatic active protein may be induced by estradiol (E_2) and nonylphenol (NP) [34].

Cat L is a cysteine protease involved in a number of physiological processes: cellular proliferation, spermatogenesis, embryo implant, yolk formation, tumour cell growth and the spread of metastases [29,35–37]. The synthesis and secretion of this enzyme are regulated by hormones and growth factors. Its gene expression is influenced by many factors, such as progesterone and estradiol [38–40]. There is recent evidence to show that in the winter flounder, *Pleuronectes americanus*, 4-NP increases Cat L gene expression [41].

The aim of this study was to obtain new information on the lysosomal enzymes Cat D and Cat L, in the Mediterranean mussel, *Mytilus galloprovincialis*. Secondly, it is to test in a preliminary way if Cat D and Cat L enzymatic activity can be enhanced in response to estrogenic compounds, such as NP and E_2; the former, a breakdown product of alkylphenol polyethoxylates (APES), was chosen for its well-documented estrogenic activity [42–45].

2. Materials and methods

2.1. *Chemicals*

E_2, haemoglobin and other chemicals were purchased from Sigma (S. Louis, MO, USA). NP was obtained from Fluka (Buchs, Switzerland); it is a mixture of isomers with differently branched nonyl side chains and contains approximately 85% *p*-isomers. The major impurities are 2-nonylphenol (*o*-isomers), dodecylphenol and dinonylphenol, which together comprise approximately 10% of the NP mixture.

2.2. *Mussel collection*

Mussels (*Mytilus galloprovincialis*) were collected from the unpolluted area of Portonovo (Ancona, Northern Adriatic Sea), at a depth of 3 m, in three different periods of the year in order to evaluate if Cat D enzymatic activity had seasonal variability (29 November 2004, 18 April 2005, 10 June 2005). The average shell length ranged between 6 and 7.5 cm. Digestive glands and gonads were rapidly excised from ten mussels, frozen in dry ice, and stored at −80 °C until processing. Mussels collected in June 2005 were used to evaluate the effect of captivity on Cat D and Cat L enzymatic activity and for the NP and E_2 exposure experiments.

2.3. *Tissue preparation*

Gonads and digestive glands were individually homogenised at 4 °C with a rotor-stator homogeniser (Ika-Ultra-Turrax T25, Janke & Kunkel) in two volumes of deionised water. The homogenate was centrifuged at 14,000 *g* for 15 min at 4 °C. The supernatant was carefully separated from the lipid layer, aliquoted and stored at −80 °C for later analysis. The total protein level in the supernatant was determined by the method of Bradford (1976) [46] with bovine serum albumin (BSA, Sigma) as the standard. Supernatants were used for enzymatic assays.

2.4. *Cathepsin D enzymatic assay*

The enzymatic activity of Cat D, in the crude extracts of digestive gland and gonad, was measured according to the methods described in Takahashi and Tang (1981) [47], with little modification, using haemoglobin as substrate. This assay utilised the hydrolytic action of Cat D on the haemoglobin molecule. Tests for the optimisation of pH, temperature and incubation time were performed on digestive gland crude extract. The final assay conditions were the following: 1 ml of incubation mixture contained 0.25 M formate buffer, pH 3.2, 0.5% haemoglobin, 25–50 µl of crude extract. Samples were incubated for 40 min at 37 °C. The reaction was stopped by the addition of 500 µl 10% trichloroacetic acid. Tubes were centrifuged at 14000 *g* for 10 min. Enzyme activity was measured in an aliquot of the supernatant, reading its absorbance at 280 nm. Cat D specific activity was expressed as unit per micrograms of proteins (U/mg), one unit of enzymatic activity is defined as the amount of enzyme producing a value of absorbance, read at 280 nm, of 1, after an incubation of 40 minutes at 37 °C, using a saturated solution of haemoglobin as substrate.

2.5. *Cathepsin L enzymatic assay*

The enzymatic activity of Cat L was routinely assayed against the synthetic substrate Z-Phe-Arg-NnapOMe (alpha-N-benzyloxycarbonyl-L-Phe-L-Arg-4-methoxy-beta-naphthylamide)

(5 mM final concentration) by a selective colorimetric assay. For the quantitative analysis we used a standard curve based on 4-Methoxy-2-Naphtylamine (0.5–35 μM), in order to convert absorbance in molar concentration. The colorimetric assay was performed as follows: 10–20 μl of digestive gland homogenate, diluted 1:50 with deionised H_2O, and 4–8 μl of gonadic tissue crude extract, were added to 376 μl of activator buffer (0.1 M NaAc, 1.33 mM EDTA, 6.66 mM cisteyn, 5.33 M urea; pH 5). The mixture was incubated for 5 min at 35 °C in order to activate the enzyme. Then were added 6.25 μl of substrate Z-Phe-Arg-NNapOMe (6 mg/mL in DMSO) and water until a final volume of 500 μl. After 20 min of incubation at 35 °C, the reaction was stopped with 500 μl of colour reagent, containing Fast Garnet Salt (1 mg/ml): pCMB (10 mM)/EDTA (50 mM), in a ratio of 1 to 1, pH 6.0. After the add of 1 mL of But-OH, the tube was centrifuged for 5 min at 14000 *g*, in order to separate the reaction product (4-Me2NA). The supernatant was read at 520 nm [48]. The assay activity was expressed as μmol/min/mg/ml of 4-Methoxy-2-Naphthyilamine released.

2.6. *Inhibition studies*

To test the enzymatic assay specificity, crude extracts for the Cat D assay were preincubated for 5 min at 37 °C with 0.1 μM pepstatin A, a Cat D inhibitor, and the residual activity was measured by the method described above. Crude extracts for Cat L were preincubated for 1 min at 35 °C with various concentrations of both leupeptin (15, 30, 60, 100 μM) and N-(benzyloxycarbonyl)-L-phenylalanyl-L-tyrosinal (Z-Phe-Tyr-CHO) (1.5 and 10 μM), two Cat L inhibitors, and the residual activity was detected by the colorimetric assay described above.

2.7. *Cathepsins enzymatic assay optimisation*

Assays at different pH values, temperatures, substrate concentrations and incubation times were carried out to establish the most appropriate assay conditions. The end goal was to obtain a set of conditions which would best reflect the overall optimal activity of Cat D and Cat L in the mussel *Mytilus galloprovincialis*. For each enzyme, all the reactions were stopped in the same way, as described above.

2.7.1. *pH*

Cat D assays were carried out at different pH values in a range of 2–5, incubated at 37 °C for 30 min, using 5% haemoglobin as substrate. For Cat L, the pH values used were in a range of 4–6; the reaction mixture was incubated for 5 min at 35 °C to allow the enzyme activation; after this step, 6,25 μl of substrate Z-Phe-Arg-NNapOMe (6 mg/ml in DMSO) were added; after 20 min of incubation at 35 °C, the reaction was stopped.

2.7.2. *Temperature*

For the Cat D assay, the reaction mixtures containing the digestive gland crude extract were incubated at different temperatures (10–50 °C), at pH 3.2 with 5% haemoglobin as substrate, for 30 min. For the Cat L assay, the temperature used was in the range 20–60 °C, pH 5 for 20 min of incubation.

2.7.3. *Incubation time*

Both Cat D and Cat L reaction mixtures were incubated for different time intervals in order to assess the time needed to reach the reaction plateau.

2.8. *Exposure to NP and E_2*

Three groups of mussels were put in a glass aquarium divided in three watertight compartments (1 mussel/l density) with artificial seawater (salinity approx. 35‰) and sufficient aeration at 20 °C with a natural light-dark cycle. Mussels were acclimatised for 7 days before exposure to chemicals. Experiments were conducted under static conditions and the chemicals were dosed only once, on day one. The first group was exposed to NP (300 μg/l) and the second group to E_2 (1000 μg/l). Both chemicals were dissolved in ethanol; the final ethanol concentration in the tanks was 0.0034%. Ethanol was added to the control group, with the same final concentration of the other tanks. The actual concentration of chemicals was not measured. Therefore, only nominal concentrations are indicated in the following text. Mussels were fed with *Chlorella* spp. colture (1×10^6 cell/l) and after 7 and 14 days, in order to assess any persistent effect, six mussels from each group were randomly collected and tissues (digestive gland and gonads) were rapidly excised, frozen in dry ice, and stored at −80 °C until processing.

2.9. *Statistics*

Data were tested for normality using the Kolmogorov-Smirnov test, and one-way analysis of variance (ANOVA) followed by Dunnett's test was performed on measured variables, with significance set at $p < 0.05$. Data are presented as means ± SD.

3. Results

3.1. *Optimisation of cathepsin D enzymatic assay*

The hydrolysis of haemoglobin by mussel Cat D was pH dependent, with the optimum at 3.2. Cat D activity exhibited a broad temperature range, with an optimum at 37 °C. The reaction reached a plateau after 40 minutes of incubation; the enzymatic activity was completely inhibited by pepstatin 0.1 μM (Figure 1).

3.2. *Optimisation of cathepsin L enzymatic assay*

The optimisation of Cat L colorimetric assay was performed using as specific substrate Z-Phe-Arg-NnapOMe and as standard 4-Methoxy-2-Naphtylamine. The assay activity was expressed as μmol/min/mg/mL of 4-Methoxy-2-Naphtylamine. The pH and temperature optimum resulted as 5 and 35 °C respectively; the reaction reached the plateau after 20 min of incubation and was completely inhibited by Z-Phe-Tyr-CHO 10 μM and leupeptin 100 μM (Figure 2).

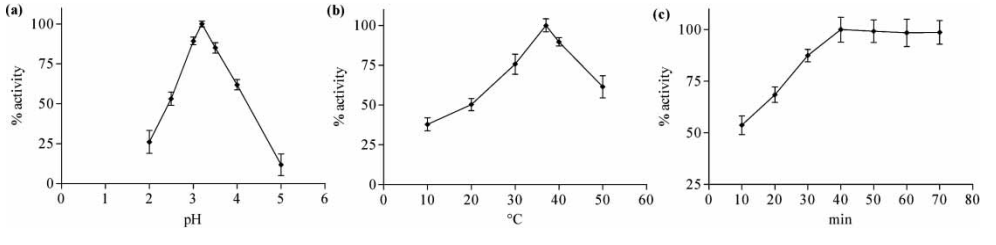

Figure 1. Cathepsin D condition assay optimisation: (a) pH, (b) temperature and (c) reaction time (mean values ± standard deviation, $n = 3$).

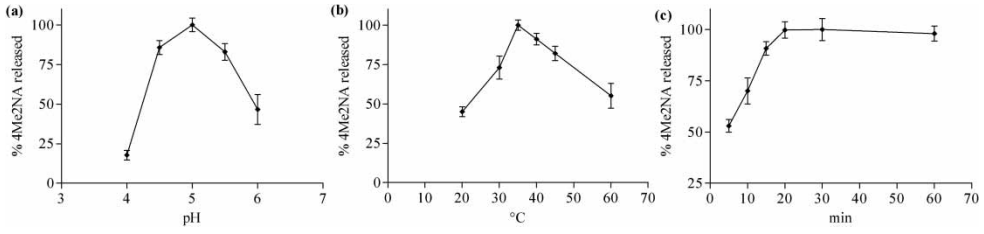

Figure 2. Cathepsin L condition assay optimisation: (a) pH, (b) temperature and (c) reaction time (mean values ± standard deviation, $n = 3$).

3.3. *Seasonality of cathepsin D enzymatic activity*

Mussels sampled in three different periods of the year showed a high seasonal variability in Cat D enzymatic activity in the digestive gland that is summarised in Figure 3. Samplings were carried out in November (29 November 2004), April (18 April 2005) and June (10 June 2005). Cat D activity exhibited a peak during the Spring period, while the minimum value was registered in November.

3.4. *Effect of captivity on cathepsin enzymatic activity*

During the three weeks of captivity, Cat D activity increased significantly after the third week in the digestive gland, meanwhile a significant decrease was observable after the third week in the gonadic tissues ($p < 0.05$) (Figure 4). Unlike Cat D, Cat L activity in the digestive gland of the control group was not influenced by captivity, while there was a significant decrease after three weeks in the gonads ($p < 0.05$) (Figure 5).

3.5. *Effect of NP and E_2 on cathepsin D enzymatic activity*

Cat D enzymatic activity in treated mussels showed significant variations after exposure compared to control groups (Figure 6). In the digestive gland of NP treated mussels, Cat D activity increased significantly after the first week of exposure ($p < 0.05$), and then rose further after two weeks. The

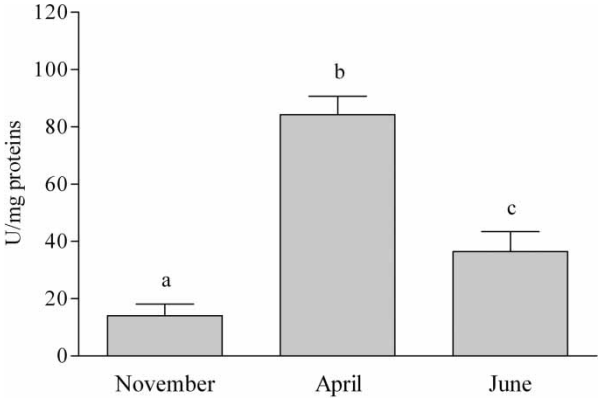

Figure 3. Cathepsin D activity in the digestive gland of mussels, *Mytilus galloprovincialis*, sampled in three different periods of the year (29 November 2004, 18 April 2005, 10 June 2005) from the unpolluted area of Portonovo (Ancona, Italy) (mean values + standard deviation, $n = 10$). Different letters indicate significant differences between group of means ($p < 0.05$).

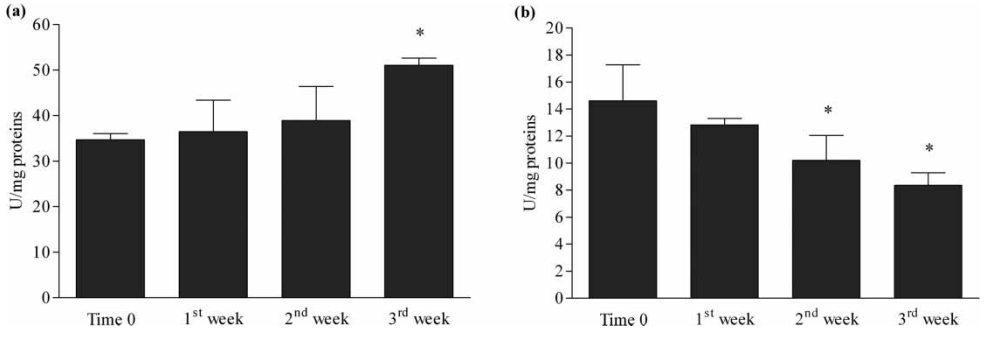

Figure 4. Cathepsin D activity (a) in the digestive gland and (b) in the gonad of mussels, *Mytilus galloprovincialis*, sampled after 1 h from the harvest (T_0) and after 1, 2 and 3 weeks of maintenance in the aquarium without any treatment (mean values + standard deviation, $n = 6$). The activities are expressed as U/mg, a unit (U) of enzymatic activity is defined as the amount of enzyme that produces at 280 nm an absorbance value of 1.0 after 40 min of incubation at 37 °C in the presence of a haemoglobin-saturating solution as substrate [29]. Asterisks (∗) indicate significant differences ($p < 0.05$) from the value of T_0.

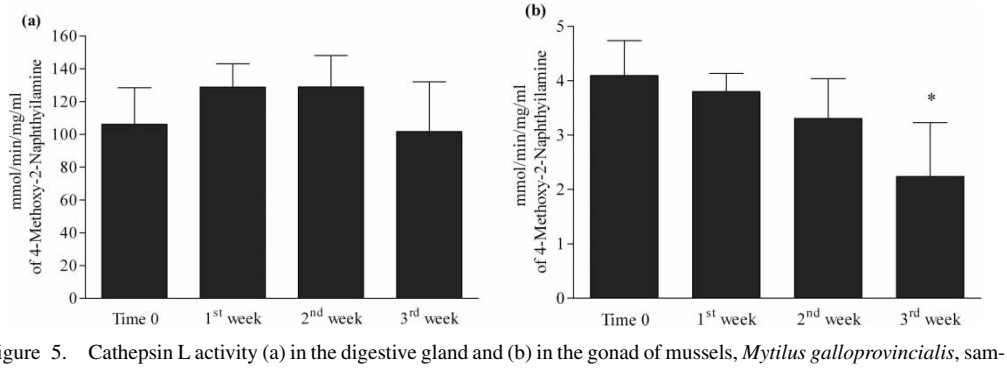

Figure 5. Cathepsin L activity (a) in the digestive gland and (b) in the gonad of mussels, *Mytilus galloprovincialis*, sampled after 1 h from the harvest (T_0) and after 1, 2 and 3 weeks of maintenance in the aquarium without any treatment (mean values + standard deviation, $n = 6$). The activities are expressed as μmol/min/mg/ml of 4-Methoxy-2-Naphthylamine. Asterisks (∗) indicate significant differences ($p < 0.05$) from the value of T_0.

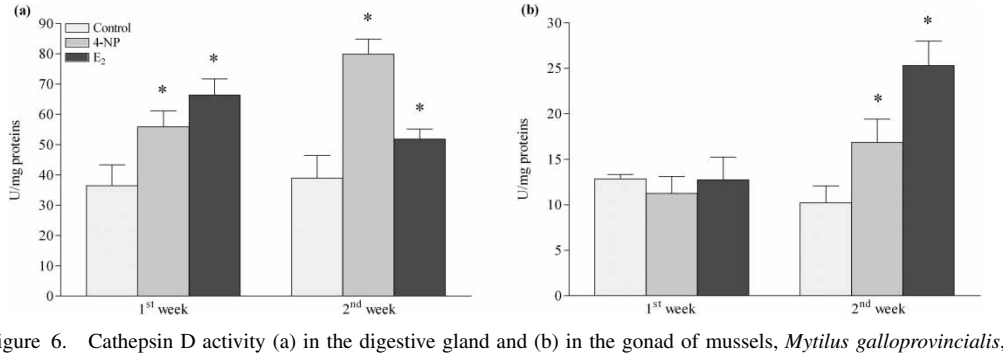

Figure 6. Cathepsin D activity (a) in the digestive gland and (b) in the gonad of mussels, *Mytilus galloprovincialis*, exposed to nominal waterborne nonylphenol (300 μg/l) and 17β-estradiol (1 mg/l) and sampled after 1 and 2 weeks after exposure (mean values + standard deviation, $n = 6$). The activities are expressed as U/mg, a unit (U) of enzymatic activity is defined as the amount of enzyme that produces at 280 nm an absorbance value of 1.0 after 40 min of incubation at 37 °C in the presence of a haemoglobin-saturating solution as substrate [29]. Asterisks (∗) indicate significant differences ($p < 0.05$) from the control value of each group.

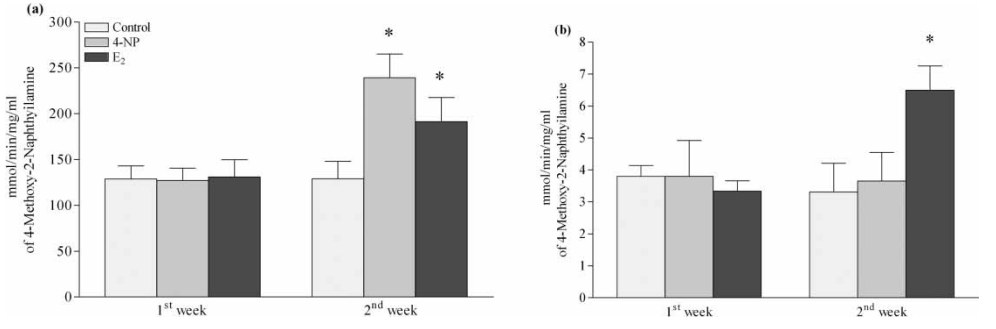

Figure 7. Cathepsin L activity (a) in the digestive gland and (b) in the gonad of mussels, *Mytilus galloprovincialis*, exposed to nominal waterborne nonylphenol (300 μg/l) and 17β-estradiol (1 mg/l) and sampled after 1 and 2 weeks after exposure (mean values + standard deviation, $n = 6$). The activities are expressed as μmol/min/mg/ml of 4-Methoxy-2-Naphthylamine. Asterisks (∗) indicate significant differences ($p < 0.05$) from the control value of each group.

group exposed to E_2 showed a significant increase in Cat D activity after the first week ($p < 0.05$), but the difference compared with the control decreased after the second week (Figure 6a). In the gonad of mussels treated with both NP and E_2, Cat D activity became significantly higher compared with the control group only after the second week after exposure ($p < 0.05$) (Figure 6b).

3.6. *Effect of NP and E_2 on cathepsin L enzymatic activity*

In the digestive gland of mussels exposed to both NP and E_2, Cat L activity showed a significant increase in activity only after the second week ($p < 0.05$) (Figure 7a). NP did not affect Cat L activity in the gonad, while in mussels exposed to E_2, a significant increase occurred after the second week ($p < 0.05$) (Figure 7b).

4. Discussion

The aim of this study was to obtain new information on two lysosomal enzymes, Cat D and Cat L, in the Mediterranean mussel, *Mytilus galloprovincialis*. Secondly, it was to test in a preliminary way if Cat D and Cat L enzymatic activity can be enhanced in response to estrogenic compounds, such as E_2 and NP.

Mussels kept in captivity showed an increase in Cat D activity over the three weeks in the digestive gland and a contemporary decrease in the gonads. On the other hand, Cat L activity decreased after the third week of captivity only in the gonads, while it did not vary in the digestive gland. This effect may be linked to the diet used during the experiment. We can therefore hypothesise that in *M. galloprovincialis*, Cat D may be involved in energetic processes, in particular in the mobilisation of proteins, stored especially in the digestive gland. Mussel digestive glands have a central role in metabolism through intra- and extracellular digestion of food particles, and distributions of nutrients to reproductive tissues in order to fill the high energetic demands associated with reproduction [49,50]; furthermore digestive cells have a highly developed lysosomal vacuolar system [51,52].

A further fact that came to light during this study was the strong seasonality of Cat D activity in the digestive gland of wild mussels. Although it is a preliminary assessment, this datum underlines that seasonality in Cat D activity is likely to occur, thus it should be explored more in depth with further seasonal sampling, and taking into account experiments involving wild mussels. The peak of activity registered in the Spring in wild mussels may be contemporary with the development and

maturation of gonadal tissue, preparation to lay gametes and spawning [53]. In other bivalves, levels of muscle carbohydrates and digestive gland proteins drop during periods of maximal gonadal development, suggesting that such a process was supported by these reserves [54]. This period requires a considerable use of reserve macromolecules, in order to obtain energy to remodel existing tissues and to create *ex novo* gonadal tissue [55,56]. The minimum activity recorded in wild mussels at the end of November could coincide with the start of gametogenesis [53]. An intermediate level of activity observed in June could coincide with the spawning/post-spawning period which is characterised by the release of gametes into the water and, subsequently, by the emptying and re-absorption of gonadal tissue [53]. The seasonality observed for Cat D enzymatic activity allows us to hypothesise a relationship with the reproductive cycle, which, together with the increase in activity observed during captivity, confirms the involvement of this enzyme in the many processes linked to the physiological state of the animal and in the mobilisation of reserve macromolecules.

Digestive glands of mussels exposed to NP, compared with the control, showed a significant increase in Cat D activity one week after exposure, persisting after two weeks. This result agrees with the starting hypothesis of a Cat D induction after exposure to estrogenic compounds. On the other hand, the increase of Cat D activity in the gonad only after two weeks from the exposure event is not easily explainable. In mussels, the digestive gland is the main target organ of pollutants, where, according to their nature, pollutants can be metabolised and accumulated [57–59]; some of the pollutants are subsequently transported to other tissues, such as the gonad, through the hemolymph [60]. However, the sampling time used during this experiment cannot clarify the exact time course of Cat D induction.

In mussels treated with E_2, Cat D response in the digestive gland confirmed the initial hypothesis of activation. However, two weeks after exposure, Cat D activity is still significantly higher than the control group, but also significantly lower than Cat D activity in mussels exposed to NP. It must be remembered that E_2 is naturally present in *M. galloprovincialis* and has an important role in sexual differentiation, gonad development and oocytes maturation [61]. Many studies suggest its involvement in cell communication. In particular, there is evidence to show that E_2 induces fast changes in hemocyte shape, causes lysosomal membrane destabilisation and the release of hydrolytic enzymes, and affects immune functions by means of pathways involving tyrosinkinase [62]. Furthermore, Janer et al. (2005) observed that, in *M. galloprovincialis*, E_2 supplied at low concentrations behaves as an endogenous steroid regulating physiological responses, for example by inhibiting aromatase-P450 and stimulating gamete maturation, while at higher concentrations it significantly induces palmitoil-CoA-estradiol-acetiltransferase, the main enzyme that inactivates an excess of E_2 in the hepatic tissue [63]. Some authors suggest the presence of different mechanisms allowing the mussel to keep stable hormone levels, for example, the conjugation of steroids with fatty acids which transforms the steroids into a non-polar form, blocking them in the lipid matrix and lowering their bioavailability [64]. This scenario could explain Cat D activity lowering two weeks after exposure, while NP, a persistent organic pollutant, may accumulate in tissues of *Mytilus galloprovincialis* and is not, or is only partially, inactivated, metabolised or eliminated from tissues [65,66], explicating its action for a longer period.

Considering the Cat D activation recorded, further experiments are needed to confirm if the NP and E_2 action mechanism is the same as in vertebrates, in which Cat D gene transcription is enhanced through the activation of the ERE sequence at the promoter region of this gene, thanks to the complex pollutant/estrogen receptor [67] and, at the same time, if it causes an increase of Cat D enzymatic activity [34]. On the other hand, it is not possible to exclude other mechanisms of action. For example, it has been observed that activation of the exchanger Na^+/H^+ in *Mytilus galloprovincialis* gonad cells due to E_2 causes the intracellular pH to vary [68]; such a change could influence Cat D and Cat L activity, which are strongly pH dependent.

Results obtained from the analysis of Cat L activity are not clear. When an effect was present, it was registered only after two weeks from the exposure event. Both NP and E_2 caused a Cat L increase after two weeks, while in the gonad E_2 but not NP had an effect. In a recent study, mussels injected with different E_2 concentrations did not show any significant variation in Cat L gene expression in the digestive gland [69]. Perhaps the delayed Cat L activation follows from other toxic effects caused by NP and E_2 exposure.

5. Conclusion

In conclusion, this preliminary study provides new information on Cat D and Cat L activity in *Mytilus galloprovincialis*. Cat D activity assayed in wild mussels in three different periods of the year suggest that a potential seasonality, probably linked to the reproductive cycle, should be taken into account and deeply investigated in the case of experiments involving wild mussels. Captivity seems to influence Cat D and Cat L activity; this effect may be linked to the diet used during the experiment, suggesting, together with the strong seasonal variation of Cat D activity, the involvement of this enzyme in metabolic processes. Furthermore, a strong Cat D induction by NP and E_2 in the digestive gland has been registered, confirming the hypothesis of Cat D activation by estrogenic compounds. All these data provide a starting point for further studies, in order to evaluate if the assay of Cat D in mussels may supply indications about the presence of estradiol-like chemicals in the environment, as happens in vertebrates.

Acknowledgements

This study was supported by a 'Fondi di Ateneo 2004, Università Politecnica delle Marche' grant awarded to O. Carnevali.

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