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Responses of Lysosomes in the Digestive Cells of the Common Mussel, *Mytilus edulis*, to Sex Steroids and Cortisol

Michael N. Moore, David M. Lowe and Pauline E.M. Fieth*

NERC Institute for Marine Environmental Research, Plymouth, United Kingdom

Summary. Estradiol-17β and progesterone at physiological concentrations in vivo induced a reduction in lysosomal stability in the digestive cells of *Mytilus edulis*. Estradiol-17β (10^{-8} M) also reduced lysosomal stability within 15 min in vitro. Lysosomal stability was determined cytochemically as the labilisation period for latent N-acetyl-β-hexosaminidase and this was shown to be inversely related to microdensitometric measurements of staining intensity for this enzyme. Estradiol-17β did not appear to induce complete labilisation or cytochemical activation of lysosomal hexosaminidase and a second, much longer labilisation period could be determined for this hormone. The effects of estradiol-17β were partially counteracted by cortisol (10^{-2} M). There was an increase in PAS staining of secondary lysosomes and an increase in alcian blue staining of residual bodies in digestive cells of animals exposed to estradiol-17β, while no changes could be observed in basophil cells. The significance of these results is discussed in terms of the physiological role of digestive cells and their possible function as target cells for estradiol-17β and progesterone.

Key words: Sex steroids – Lysosomal stability – Digestive cells – *Mytilus edulis* – Target Cells.

Szego (1975) has reviewed the reduction of lysosomal stability and release of hydrolases by steroid and polypeptide hormones. Lysosomal destabilisation has also been implicated in stress responses in *Mytilus edulis* (Moore, 1976; Bayne et al., 1976) which indicate that this type of mechanism is induced by a number of physical

Send offprint requests to: Dr. M.N. Moore, Institute for Marine Environmental Research, Prospect Place, The Hoe, Plymouth PL1 3DH, England

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stressors as well as by polycyclic aromatic hydrocarbons (Moore and Lowe, unpublished results). In view of the importance of lysosomes in the molluscan digestive cell (Summer, 1969; Owen, 1970, 1973) and their function in autophagic utilisation of reserves (Moore and Halton, 1973, 1977; Yoshino, 1975; Bayne et al., 1976; Thompson et al., 1974), an investigation has been initiated to examine steroid action in relation to lysosomal function in *Mytilus*. The digestive cells of *Mytilus* are known to act as a site for the storage of reserves for use in gametogenesis (Thompson et al., 1974) and estradiol-17β and testosterone have been demonstrated at certain stages of this process (Longcamp et al., 1974).

The present investigation involved the use of a cytochemical test for lysosomal stability (Moore, 1976), microdensitometry and light microscope cytology to examine the responses of digestive cell lysosomes in *Mytilus* to a number of sex steroids and cortisol.

Materials and Methods

Mussels (*Mytilus edulis*) 50–60 mm in length were collected from the River Lynher at Plymouth and acclimated for 12 days at 16° C for replicate I and III, and one day at 16° C for replicate II.

Injections of 100 µl 5×10^{-7} M estradiol-17β (Sigma), progesterone (Sigma) and testosterone (Sigma) respectively in sterilised filtered synthetic seawater (HW marinemix containing 0.005% ethanol) were made into the posterior adductor muscle with a 26 gauge hypodermic. Synthetic seawater +0.005% ethanol was used as the vehicle control. A 100 µl suspension of 10^{-2} M hydrocortisone (cortisol) hemisuccinate (Sigma) in synthetic seawater was injected by the same route with a synthetic saline cortisol vehicle control. Estradiol-17β (5×10^{-7} M) plus cortisol (10^{-7} M) was injected in 100 µl synthetic seawater containing 0.005% ethanol. Five mussels per replicate were employed except where otherwise stated. Mussels were sacrificed after 2 h and the digestive glands rapidly excised.

In vitro experiments employed slices of digestive gland (approximately 3–4 mm thick), which were subsequently incubated with either vehicle control or estradiol-17β (10^{-6} M and 10^{-8} M) for 15 min at 20° C. This was followed by a rapid rinse in filtered synthetic seawater (4° C) prior to cytochemical processing.

Small pieces of digestive gland (approximately 5 mm)^3 were frozen in hexane at −70° C and sectioned (10 µm) in a Bright Cryostat at a cabinet temperature of −26° C using dry ice to cool the knife (Bitensky et al., 1973). Labilisation periods of N-acetyl-β-hexosaminidase were determined in tissue sections of digestive gland as previously described (Moore, 1976), with pre-incubation from zero to twenty-five minutes at five-minute intervals. Microdensitometric measurements of hexosaminidase activity were made in the mid-region of digestive cells (10 readings/section) with a Vickers M85 scanning integrating microdensitometer at a wavelength of 540 nm with mask size A2, slit-width setting of 60 and a ×40 objective. Some measurements were also made with a ×100 objective (oil immersion) to check for distributional error; however, ratios of relative absorbance for different experimental conditions were not significantly different from ratios determined with a ×40 objective.

Pieces of digestive gland were fixed for cytological examination in a modified Baker's calcium formal (+2.5% NaCl) for 24 h at 4° C, then stored in gum-sucrose until alcoholic dehydration and wax embedding. Sections (5 µm) were stained by the Papanicolaou (Culling, 1963) and alcian blue-PAS (Pearse, 1968) techniques.

Statistical analyses of the experimental results were made using the parametric F-test (one-way analysis of variance) and the non-parametric Mann-Whitney U-test. Probability values given in the text and in tables are those determined from the parametric F-test. However, where results were not also found to be significant ($P \leqq 0.05$) with the non-parametric U-test, the exact probability value obtained is quoted. The analyses were performed on untransformed data (ie. labilisation period in minutes) and not on data transformed to percentage of vehicle control, although this latter format is used in two tables and the text for comparative purposes.

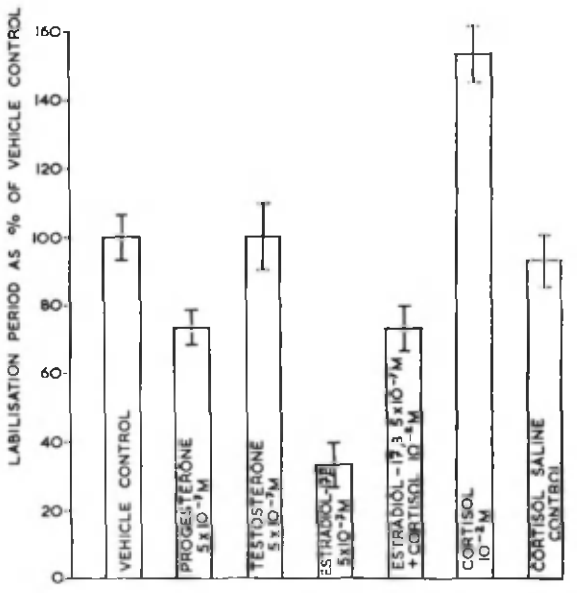


Fig. 1. The effects of injections of sex steroids (100 µl 5×10^{-7} M) and cortisol (100 µl 10^{-2} M) on the labilisation period of lysosomal hexosaminidase after 2 h in *Mytilus edulis* (Mean ± SE)

Results

Cytochemistry

There were significant reductions in the labilisation period for hexosaminidase in the digestive cells of mussels sampled 2 h after injection with 100 µl of estradiol-17β (5×10^{-7} M; $P < 0.001$) and progesterone (5×10^{-7} M; $P < 0.01$) respectively (Fig. 1). Replicate experiments indicated reproducibility of the lysosomal response to both hormones (Table 1). The injection of 100 µl of testosterone (5×10^{-7} M), however, did not have any significant effect on the labilisation period of hexosaminidase (Fig. 1).

The injection of 100 µl of a suspension of hydrocortisone (cortisol) hemisuccinate (10^{-2} M) induced a significant increase in labilisation period (153% ± 8.16, Mean ± S.E., $P = 0.01$) (Fig. 1), while the saline (cortisol vehicle) control was not significantly different from the steroid vehicle control (0.005% ethanol in saline). A combination of estradiol-17β (5×10^{-7}) and cortisol (10^{-2} M) gave a labilisation period of 73.4% ± 13.03 (Mean ± S.E.; Number of animals = 4) which was significantly higher than estradiol-17β alone (33.3% ± 6.73, Mean ± S.E., $P < 0.01$).

A secondary increase in staining intensity was observed in estradiol-17β injected mussels with longer pre-incubation (labilisation) times (Table 1). This effect has also been observed in some field samples (Moore, unpublished results) and was further encountered in a third vehicle control replicate, although here the secondary

Table 1. The effects of estradiol-17β and progesterone on the labilisation period of hexosaminidase in the digestive cells of *Mytilus edulis*

Treatment		Labilisation period as % of vehicle control (Mean ± SE)	
Vehicle control	I	100 ± 5.27	
	II	100 ± 6.65	
Estradiol-17β (100 µl 5×10^{-7} M)	I	36.8 ± 6.44 (Primary, $P < 0.001$);	131.2 ± 5.32 (Secondary, $P < 0.01$)
	II	33.3 ± 6.73 (Primary, $P < 0.001$);	126.7 ± 6.66 (Secondary, $P < 0.05$, $P = 0.075$ Mann-Whitney U-Test)
Progesterone (100 µl 5×10^{-7} M)	I	73.7 ± 5.27 ($P < 0.01$)	
	II	66.7 ± 6.73 ($P < 0.01$)	

Table 2. The effects of estradiol-17β and cortisol on the labilisation period and staining intensity (absorbance) of lysosomal hexosaminidase in the digestive cells of *Mytilus edulis*

Experimental treatment	Labilisation period (min) ± SE	Absorbance of hexosaminidase staining reaction ± SE
Vehicle control	15.0 ± 1.01	32.2 ± 4.70
Estradiol-17β (5×10^{-7} M)	5.0 ± 1.01	65.3 ± 4.94
Cortisol (10^{-2} M) + Estradiol-17β (5×10^{-7} M)	13.8 ± 1.25 (4)[a]	25.5 ± 3.15 (4)
Cortisol (10^{-2} M)	23.0 ± 1.22	29.0 ± 4.85
Cortisol saline control	17.5 ± 1.44 (4)	35.6 ± 6.65 (4)
Progesterone (5×10^{-7} M)	11.0 ± 1.02	58.0 ± 7.19

[a] Number in parentheses is number of animals in experimental condition

increase in staining intensity was less pronounced than with estradiol-17β. The labilisation period for this secondary increase in the controls was 166.7% ± 6.73 (Mean ± S.E.) of the primary labilisation period.

Microdensitometric determinations of staining reaction for hexosaminidase, made on sections after five minutes pre-incubation at pH 4.5, showed a significant increase in absorbance ($P < 0.01$) from the control condition after treatment with estradiol-17β (5×10^{-7} M) (Table 2; Fig. 2A, B) and progesterone (5×10^{-7}) (Table 2; $P < 0.001$). Staining intensity was significantly lower ($P < 0.001$) when a combined dose of cortisol (10^{-2} M) and estradiol-17β (5×10^{-7} M) was injected into mussels (Table 2; Fig. 2C), than with estradiol-17β alone. Regression analysis of absorbance values for hexosaminidase and labilisation periods in five experimental conditions (Table 2) showed a significant inverse linear relationship between these two parameters ($r = -0.64$; $P < 0.001$ with 25 degrees of freedom). The inference from this result is that a decrease in labilisation period is directly related to increased lysosomal hexosaminidase activity.

In vitro incubation of tissue slices of digestive gland for 15 min with 10^{-6} M estradiol-17β induced a significant reduction in the labilisation period of

Table 3. The in vitro effects of estradiol-17β on the labilisation period of lysosomal hexosaminidase on tissue slices of digestive gland of *Mytilus edulis*

In vitro incubation condition (15 min)	Labilisation period as % of vehicle control ± SE
Vehicle control	I 100 ± 5.76 II 100 ± 7.19 III 100 ± 5.76
Estradiol-17β 10^{-6} M	I 60.0 ± 11.56, $P<0.05$ II 58.5 ± 8.37, $P<0.05$ III 66.8 ± 6.68, $P<0.05$
Estradiol-17β 10^{-8} M	III 53.2 ± 6.68, $P<0.01$

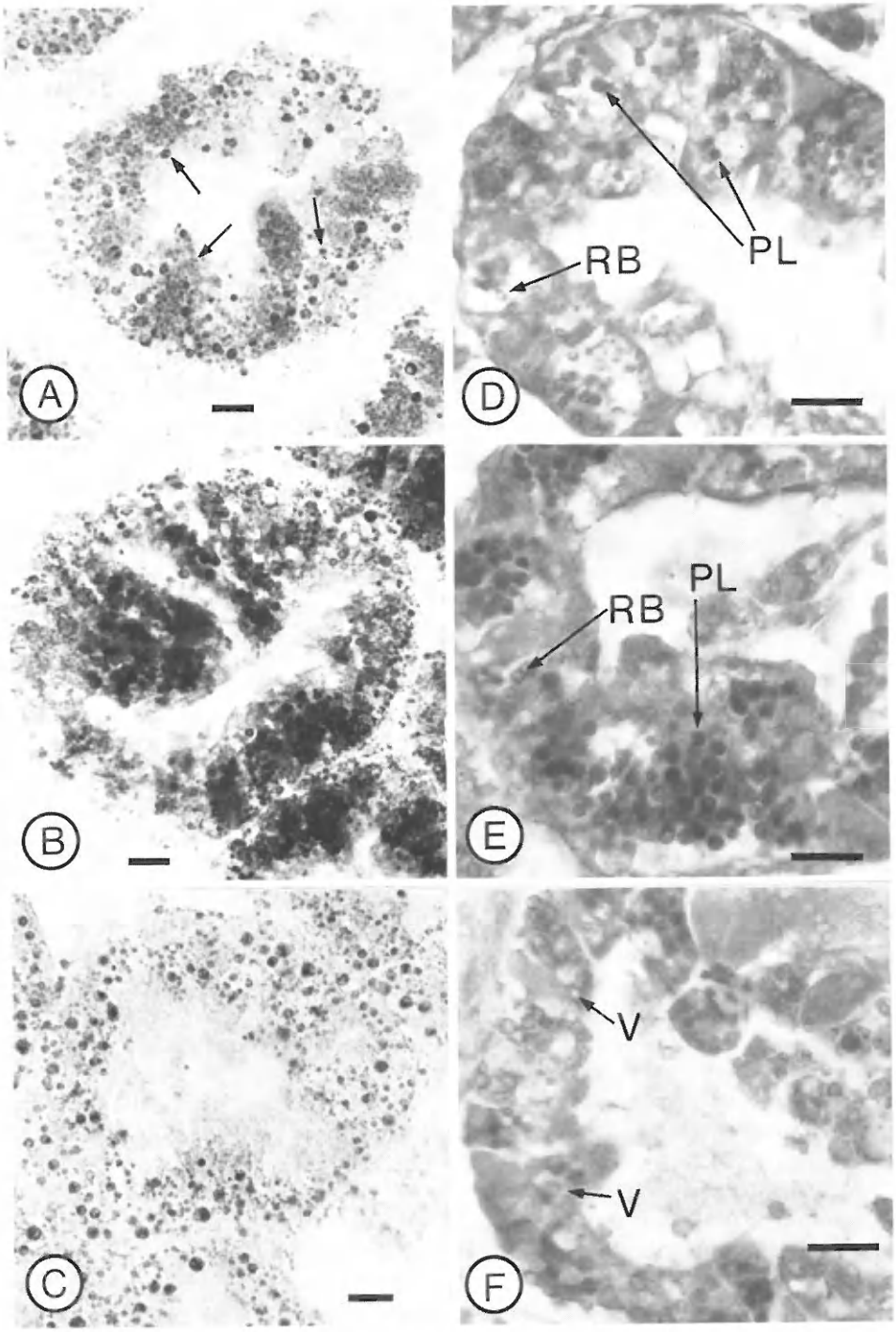
hexosaminidase (Table 3) from the vehicle control. Incubation with 10^{-8} M estradiol-17β also induced a significant reduction in lysosomal stability (Table 3).

There was no observable hexosaminidase staining reaction in basophil cells in any of the experimental conditions. The sex of only a small number of the mussels used could be accurately determined; however, the low variability of the results indicates that there was no major sexual difference in response to the hormones.

Cytology

Digestive cells from three of the experimental conditions were examined cytologically. A marked feature of animals in each of these conditions was the structural and staining consistency of the digestive cells. Digestive cells from vehicle control animals generally had apical vacuoles (3.0–3.5 μm diameter) although some cells were devoid of these structures and contained small granules (3.0 μm diameter) which usually stained green with the Papanicolaou technique, and were PAS positive with alcian blue-PAS. Three types of granules could be distinguished with the alcian blue-PAS method: 1. PAS positive granules (2–3 μm diameter), 2. granules with an amber rim which were slightly PAS positive (2 μm diameter), 3. alcian blue positive granules some of which showed a halo staining effect (1.5–2 μm diameter) (Fig. 2D). The alcian blue positive granules tended to be associated with digestive cells which had a PAS positive network, while the amber granules were usually found in the highly vacuolated digestive cells. PAS positive granules were generally associated with the alcian blue positive forms although they could also be found with the amber form.

Mussels injected with estradiol-17β showed increased vacuolation of the digestive cells with slight disruption of the luminal border and reduction in height of the tubule epithelium (Fig. 2E). There was an increase in the number of granules which stained positively with alcian blue and PAS in the digestive cells (Fig. 2E). PAS staining of the tubule epithelium tended to be very regular and apart from the presence of granules it was difficult to define the borders between pyramidal and digestive cells. All three types of granules were present; however, a fourth type intermediate between PAS positive and alcian blue positive forms was apparent (3 μm diameter). Many of the alcian blue positive granules were larger than those in the controls and were of the same size order as the intermediate type.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The digestive cells of animals injected with cortisol contained numerous small vacuoles (1–2 μm diameter) (Fig. 2F), although there was an overall reduction in numbers of the large vacuoles which were present in the controls (Fig. 2D). The luminal border showed an increase in PAS staining intensity. There was an overall increase in the size and numbers of the three types of granule (Fig. 2F); however, this increase in numbers was not as pronounced as with estradiol-17β (Fig. 2E) and the digestive cells were still similar in general appearance to the control condition (Fig. 2D).

Discussion

Longcamp et al. (1974) and Saliot & Barbier (1971) have demonstrated the presence of testosterone ($5 \times 10^{-9} - 10^{-7}$ M) and estradiol-17β (2×10^{-8} M) in the gonad of *M. edulis* and progesterone in the female gonad of *Pecten maximus* respectively. The results show that estradiol-17β and progesterone injected at physiological concentrations (Longcamp et al., 1974) specifically induce destabilisation of the lysosomes of the digestive cells of *Mytilus edulis*. This destabilising effect was also shown to be directly related to an increase in staining intensity for lysosomal hexosaminidase. Testosterone did not have any effect on lysosomal stability while cortisol, an established stabiliser of lysosomal membranes (Weissmann, 1969), induced a significant increase in lysosomal stability and partially counteracted the destabilising effect of estradiol-17β. The mussels used in this investigation were approximately two months post-spawned, the mantle tissue consisting mainly of somatic cells, a condition in which endogenous hormone levels would be expected to be minimal.

A considerable body of evidence has been accumulated to show that high levels of sex steroids can modulate lysosomal stability in vitro (Weissmann, 1969). However, Szego (1975) has reviewed the effects of a number of polypeptide and steroid hormones on the lysosomes of target cells in mammals, and concluded that in certain tissues the hormone-receptor complex was transferred to the nucleus by the endocytic-lysosomal system. This mediation also involved destabilisation of lysosomes and limited release of lysosomal hydrolases in rat preputial gland (Szego et al., 1976) and in amphibian urinary epithelial cells (Pietras et al., 1975). The release of lysosomal proteins into the cytoplasm and nucleoplasm after treatment with estradiol-17β has been demonstrated in rat preputial gland cells by immunofluorescence (Szego et al., 1977).

Fig. 2. A A digestive tubule from a vehicle control condition stained to show hexosaminidase after 5 min labilisation (pH 4.5), showing staining reaction in lysosomes (arrowed). **B** A digestive tubule from a mussel injected with 100 μl 5×10^{-7} M estradiol-17β showing an increase in the staining intensity of the lysosomes. **C** A digestive tubule from a mussel injected with 100 μl 5×10^{-7} M estradiol-17β plus 10^{-2} M cortisol showing a decrease in staining intensity from that induced by estradiol-17β alone. **D** A digestive tubule from a vehicle control condition showing PAS positive phagolysosomes (*PL*) and alcian blue positive residual bodies (*RB*). Alcian blue – PAS method. **E** A digestive tubule from a mussel injected with 100 μl 5×10^{-7} estradiol-17β showing increased staining of PAS positive phagolysosomes (*PL*) and alcian blue positive residual bodies (*RB*). Alcian blue – PAS method. **F** A digestive tubule from a mussel injected with 100 μl 10^{-2} M cortisol showing numerous small vacuoles (*V*) and a slight increase in size and numbers of granules from the vehicle control condition. Alcian blue – PAS method. Scale bar = 10 μm

The cytological changes observed in response to injections of estradiol-17β in mussels further substantiate the evidence for alterations induced in lysosomes. These changes involved the increased staining intensity of PAS positive lysosomes and alcian blue positive granules which were probably residual bodies (Owen, 1973; Wigham, 1976).

The implications of hormonally induced changes in lysosomal stability in the digestive cells of mussels are manifold. The present results indicate that the digestive cells are a target tissue for estradiol-17β which is known to be present in *Mytilus* (Longcamp et al., 1974) and may be a target tissue for progesterone, the presence of which has not been confirmed in *Mytilus*, although it can be synthesised in vitro by both male and female gonad (Longcamp et al., 1974). An alternative proposal could be that estradiol-17β and progesterone trigger the release of another endogenous hormone, perhaps neurosecretory, and that the digestive cells are the target tissue for this second hormone rather than the steroids. However, the short time course for the action of the steroids (2 h) in vivo together with the results of in vitro incubation with estradiol-17β, which showed a significant destabilisation after 15 min while eliminating the possibility of a secondary hormonal action, would tend to support the hypothesis that estradiol-17β induces a primary effect in the digestive cells. Stabilising effects of cortisol on lysosomes, described previously in *Mytilus* by Moore (1976), have been confirmed and the counteractive effects of cortisol and estradiol-17β demonstrated. The potential for limited cytolysis by the release of hydrolases from destabilised lysosomes could indicate a hormonally controlled mechanism for limited cellular catabolism. Other consequences such as changes in membrane fluidity and permeability induced by limited proteolysis may also be involved (Barnett et al., 1974; Pietras et al., 1975).

Seasonal changes in endogenous levels of estradiol-17β and possibly other hormones may be implicated in triggering catabolic processes involving lysosomal function in the digestive cells of *Mytilus*. These processes, although undoubtedly complex, may be at least partially elucidated by continuing cytochemical investigations of the type described.

This complexity is perhaps exemplified in the duality of the response to estradiol-17β where two distinct labilisation periods could be distinguished. Interpretation of this effect is difficult. However, if the cytochemical technique is measuring a genuine functional parameter then it may indicate partial destabilisation, and suggests a further mechanism in the control of estradiol-17β function in the digestive cells. The occurrence of this particular phenomenon in one of the control replicates, and in some field samples of *Mytilus* and the clam *Scrobicularia plana* (Moore, unpublished results) would further support the hypothesis that this type of cytochemically determined response may be related to normal function of the bivalve digestive gland.

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