RESEARCH ARTICLE **(Open Access)**

The impact of short-term exposure to hypoxia on Mediterranean crab *Carcinus aestuarii*

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Abstract

Hypoxia is one of the key threats to most of the marine environment. Although motile organisms have the potential to avoid the low oxygen conditions, they can suffer indirect and sub lethal impacts as a result. Little is known of how acute hypoxia affects physiology in crustaceans. The present study assesses the effect of hypoxia on the heart rate, hemolymph glucose levels and lysosomal membrane stability on Mediterranean crab, *Carcinus aestuarii*. The adult crabs *C.aestuarii*, were kept in hypoxic waters for 24 hours. Hypoxic conditions (50-60 mm Hg O_2) were induced by allowing the crabs to consume their oxygen supply. The hemolymph glucose levels, the heart rate and neutral red retention time (NRRT) as a biomarker of lysosomal membrane stability have been assayed in both treatment (intact and eyestalk-ablated animals) and control groups. The hemolymph glucose level in intact animals were significantly increased (from 38.2 ± 3.2 to 141.2 ± 15.8 mg/dL, F=9.984, df =1, 11, p=0.010 so p<0.05), while in eyestalk-ablated animals has been slightly decreased (from 27±2.4 to 24±1.8 mg/dL, F=0.993, df=1, 10, p=0.343 so p>0.05). Heart rate were significantly increased by 30 % in treatment group after exposure to hypoxic water for 2 hours. NRRT has been significantly reduced in treatment group (from 120±25.2 to 42.3±11.4 min, p<0.05) after exposure to hypoxia. Our findings highlight the importance of understanding how environmental disturbances modify the physiological stress responses of crustaceans to survival hypoxia. Quantifying the relationship between physiological responses and environmental stressors, is crucial for developing mechanistic models that can predict how changes in disturbances over time in coastal ecosystems will impact ecological processes, particularly in the context of global climate change.

Key words: hypoxia, crustacean hyperglycemic hormone(CHH), hemolymph glucose level, heart rate, lysosomal membrane stability

1. Introduction

Nowadays, global climate change is becoming a real concern all over the World. The continuous accumulation of CO_2 and temperature increasing over the average values are factors that cause global climate change [1]. Changes of environmental CO_2 and temperature may normally be concomitant of environmental hypoxia development, which can be caused in the minimum layers of oxygen in the intermediate depths, found in bottom waters which are isolated, at night in tide pools, and also in marine sediments [2, 3, 4].Oxygen depletion or hypoxia is one of the environmental stressors which effect the animals which live in aquatic ecosystems worldwide. When animals are exposed to different factors, which can affect their physiological systems, they use different stress responses in order to establish the homeostasis and when the homeostatic response is partially or completely failure, than it increases the physiological response disturbance which may lead into death of the animal [5]. Tolerance against stress by animals can effect it distribution and abundance [6, 7], which can also change disturbance probability [8,9], the population growth rate[10,11], and interaction with other species[12, 13]. Benthic invertebrates response by stress in low level of oxygen in some aquatic ecosystems [14]. The level of dissolved oxygen in water column of 2 mg O_2/L or lower,is considered hypoxic and may lead into harmful effects in animals [15]. Hypoxia may be present in different ecosystems such as coastal areas, lakes and estuaries [16].The effects of hypoxia on biotic ecosystems depends on 2 factors: how long hypoxia will durate and how much the concentration

of dissolved oxygen will decrease [17]. When hypoxia lasts for many weeks or is so close to anoxia, than the migration of motile animals may occur [18] and even the mortality of animals [19, 20].Many studies report about the mortality caused in motile animals by hypoxia [21]. When the dissolved oxygen concentration is above anoxic level or hypoxic episodes are short, hypoxia may result in change species composition [22, 23] and decrease the biomass[24]. Different species experience different tolerance values against hypoxia [25]. Species which show a relatively tolerance level are polychaetes, bivalves, platyhelminths and cnidarians, while crustaceans and vertebrates have relatively low hypoxia tolerance [26, 27].On our study we have been focused on the hyperglycemia induced by hypoxia in the Mediterranean green crab, *Carcinus aestuarii*,which is found in Narta Lagoon, Albania. Hyperglycemia is a typical response which is observed under different stress conditions in decapods [28]. If the animal is exposed to hypoxia immediately, than the glucose level will change immediately too [29]. Crustacean Hyperglycemic Hormone (CHH) which is found in crustaceans, is produced by the eyestalk X-organ and stored in sinus glands, and it regulates the haemolymph glucose level of the animal [30].This study evaluates the effects of hypoxia on heart rate, haemolymph glucose level and lysosomal membrane stability. In Albania, few studies are done in these species in order to evaluate the environmental pollution by using them as biomarker [31,32].

2.Materials and methods

2.1. Collection of animals and the experiment design

Animals were collected by a fisherman during April 2015, in Narta Laggon, in Vlora city, Albania and were placed into buckets. In order to avoid stress, animals were immediately brought in the Laboratory of Physiology and Biochemistry in the Faculty of Natural Sciences, Tirana University. Animals were acclimated for 3 days in the lab and during this period they were maintained in aquaria of 30 L filled with sea water which was continuously aerated (SNSW, Nutri-Sea Water® Aquarium Saltwater, pH: 8 ± 0.1; salinity: 36±1 ppt; and temperature of 17 ± 1°C). Animals were fed with fish once a day and algal slurry. After three days of acclimatization, 20 adult animals were choice for the experiment. Animals were placed into two containers and divided into 2 groups of 10 animals each: an intact group and eye-stalk ablated animals.Before being exposed to the low levels of oxygen, the haemolymph glucose level, the Neutral Red Retention Time and heart rate of each group were measured, in order to compare the physiological response of animals against hypoxia before and after the experiment. After that, animals were kept into close containers in order to reduce the amount of oxygen inside of it for 24 h. After 24 h the glucose level was measured again.

2.2. Collection of haemolymph and measurement of the glucose level

The haemolymph was collected at the base of the fourth moving leg.The haemolymph was with drawn very carefully through the arthrodial membrane into a 2.5 mL hypodermic syringe fitted with a 25 gauge needle and having 0.5 mL of physiological Ringer solution for crustaceans: [20 mM (4.77g) Hepes, 436 mM (25.48g) NaCl, 53 mM (13.06g) MgSO4, 10 mM (0.75g) KCl, 10 mM (1.47g) CaCl2), pH 7.4]. The obtained solution was discarded into a 2.0 mL siliconised (Sigmacote) Eppendorf tube held in ice water. In order to avoid excessive stress, animals were manipulated very carefully. Measurement of the haemolymph glucose levels in the crabs was done enzimatically and by using a portable glucometer (One Touch-Ultra).The lysosomal membrane stability was evaluated by measuring the neutral red retention time (NRRT).The cran heart rate was measured by using electromyogram (EMG) electrodes.

2.3. Neutral Red Retention Assay

Neutral Red Retention Assay was realized according to Standard Operative Procedure (SOP) proposed by Lowe et al. (1995) [33], prescribed from Martinez-Gomez et al. (2008) [34] and adopted for the specimen taken under the study. The physiological saline and neutral red stock solutions were prepared prior to the beginning of the assay. Initially, 0.5 mL haemolymph from each crab was collected with the syringes containing 0.5 mL of physiological saline solution and transferred to an Eppendorph tube. Immediately, 50 μL haemocyte cells solution was pipette and dropped onto a glass slide. The slides were placed into a dark and humid chamber and incubated for 15 min. Then, the neutral red stock solution was prepared by the dilution of 20 mg Neutral Red dye in 1.0 mL dimetilsulphoxid (DMSO). Working solution was prepared by the dilution of 5.0 μL of stock solution in 995 μL physiological solutions. After incubation, 50 μL NR working solution was dropped onto each slide. At the end of 15 min, the slides were quickly examined by microscopy. The cells were observed for the structural abnormalities and for the retention time of the neutral red dye. The slides were observed every 15 min until 50 % of the haemocytes lost the dye in the cytosol. A mean of neutral red time and glucose concentrations were calculated for each group.

2.4. Statistical Analysis

The results of each parameter obtained for each experimental group were compared among the treatment groups using either the parametric analysis of variance (ANOVA), or the nonparametric analysis (Kruskal Wallis test) based on the data distribution (normality and homogeneity of variance). When an indication of a significant difference ($p<0.05$) was observed, differences were analyzed by the post-hoc Dunn's test. Simple linear correlation (Pearson test) conducted with the mean values, was used to establish the significant relationships between the biological responses.

3. Results and discussions

Research done in *Libinia* [35] have been shown that if biologist transfer animals from sea water to the land and stay there even after one hour, their haemolymph glucose level increases drastically. This fact suggests that sinus glands are maybe involved on the glucose level regulation in haemolymph [36].If eyestalk ablated crab animals are exposed to the hypoxic conditions, there is noticed a non-significant change on the haemolymph glucose level because of the removed eyestalks which contain sinus glands.When *Carcinus aestuarii* are exposed for 24 hours to hypoxic water, a significant increasing of their haemolymph glucose level were noticed. Further more,after 2 h of hypoxic exposure, the heart rate of crab were increased by 30 % and the NRRT decreased significantly from 120±25.2 to 42.3±11.4 min. The experimental results show a significant typical hyperglycemic response on the control group (from 38.2 ± 3.2 to 141.2 ± 15.8 mg/dL haemolymph, $F=9.984$, df =1, 11, $p=0.010$ so $p<0.05$), while into eyestalk ablated animals, hyperglycemia doesn't occur but it was noticed a non-significant decreasing of the glucose level (from 27±2.4 to 24±1.8 mg/dl haemolymph, $F=0.993$, df=1, 10, $p=0.343$ so $p>0.05$). The crab haemolymph glucose levels and shown in Table 1 and Figure 1.

Table 1: Results of hyperglycemia before and after exposure to hypoxic water

Animals	*No. animals*	*Before exposure*	*After exposure*
Eyestalk ablated	10	27±2.4	24 ± 1.8^{NS}
Intact	10	38.2 ± 3.2	141.2 ± 15.8 **

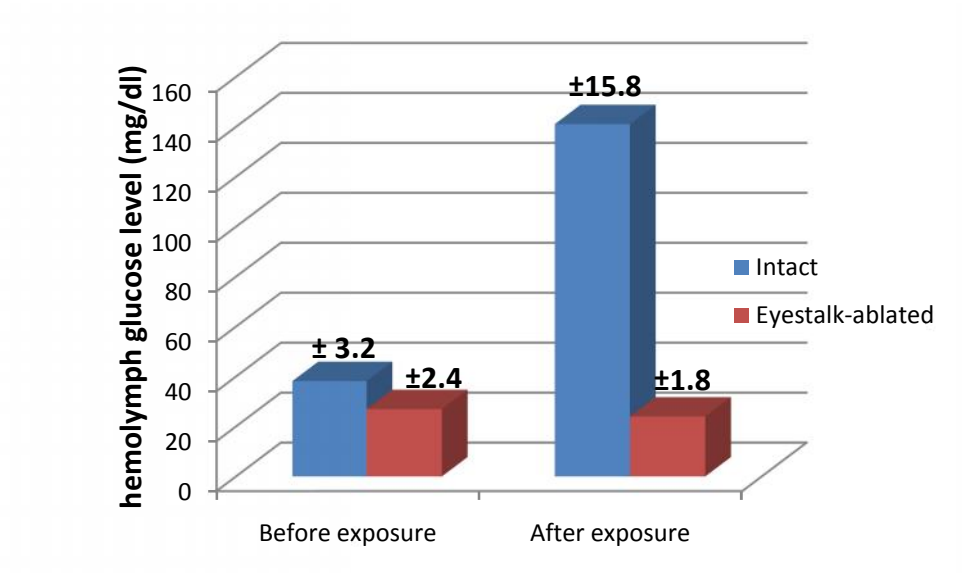

Figure 1: Glucose level in crab haemolymph (mg/dl) observed in intact and eyestalk ablated animals before and after exposure to hypoxia . The values ± SD and the results are significant ($p< 0.5$).

Hyperglycemia is not the only effect which hypoxia causes. It affects also the lysosomal membrane stability. In order to define the effects of hypoxia in the membrane stability of lysosomes, Neutral Red Retention Time was measured. One of the most important functions of lysosomes is that they serve as autophagic centers of several components of cytoplasm such as proteins and organelles [37, 38]. This process is not well known but is reported that both micro-autophagy and macro-autophagy are involved [39, 40]. Actually many factors such as membrane permeability and also hydrolases in collaboration with structural components affects the structure-linked latency of lysosomal enzymes [41]. It is well reported that many agents such drugs, diseases, hormones and stressor cause destabilization in lysosomes which causes a reduction of hydrolases latency [42, 43]. This is why this research has been focused on the alterations caused in crabs by the hypoxia as a stressor. Figure 2, shows a significant change of Neutral Red Retention Time (NRRT) into the eyestalk-ablated group after a hypoxic water exposure for 24 h.

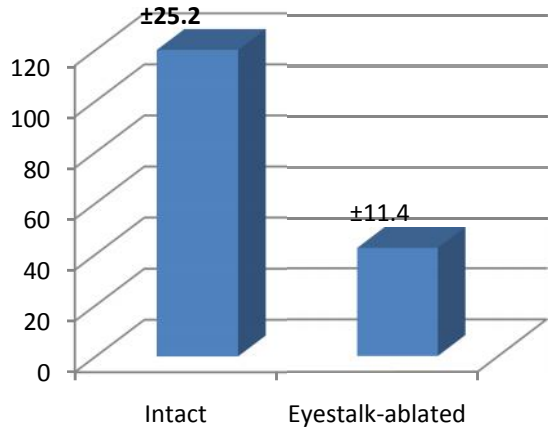

Figure 2: NRRT observed in intact and eyestalk ablated animals. The values are means ± SD and the results are significant ($p < 0.05$)

These results indicates an alteration into lysosome membrane stability. Anyway the relationship between pathological or physiological conditions and lower performance of hydrolases latency and the catabolism of proteins is not clear enough and this make difficult to define if the functional changes occurred in lysosomes, results from dysfunction of the cell [444]. In vivo experimental conditions such as hypoxia, temperature,

and salinity affects the lysosomal membrane stability [45]. A lot of biochemical procedures used in different experiments have been proved that before mentioned experimental treatment conditions caused a decrease in cytochemically determined stability of lysosomes, closely related with hydrolases latency [46]. It is reported that maybe these factors are involved in the increasing permeability of the lysosomal membrane, since that they are periodically reversible by treatment of cortisol, which is well known as a membrane stabilizer [47, 48]. Other studies should still be done in order to elucidate in more detail this process.

4.Conclusions

Nowadays, many climate changes are a real concern for all living organisms and sometimes they cause drastic changes in their physiological responses by considering them detectors of what is happening in their environment. In some species they cause even death. Hypoxia, which result by decreasing the O_2 level in oceans, in estuaries, rivers or lakes is a factor which affects animal metabolism. It causes drastic changes in the haemolymph glucose level in the Mediterranean green crab *Carcinus aestuarii*, which means that this stressor affects their carbohydrate metabolism by increasing their glucose level drastically. It also affects the lysosomal membrane stability and heart rate, which indicates that the effects of hypoxia into crustaceans are deeper than our expectations which make these species a biomarker of an early stage of hypoxia. Our findings highlight the importance of understanding how environmental disturbances modify the physiological stress responses of crustaceans to survival hypoxia. Quantifying the relationship between physiological responses and environmental stressors, is crucial for developing mechanistic models that can predict how changes in disturbances over time in coastal ecosystems will impact ecological processes, particularly in the context of global climate change.

5.References

1. Bazzar AF : **The Response of Natural Ecosystems to the Rising Global CO2 Levels.** Annual Review of Ecology and Systematics 1990, **21**:167-196
2. Knoll AK, Bambach KR, Canfield ED and Grotzinge PJ : **Comparative Earth history and late Permian mass extinction, Science** 1996, **273**: 452–457.
3. Bridges CR: **Adaptation of vertebrates to the intertidal environment, in The Vertebrate Gas Transport Cascade: Adaptations to Environment and Mode of Life:** JEP, W, Bicudo; 1993, CRC Press, Boca Raton, Fla.
4. Grieshaber MK, Hardewig I, Kreutzer U and Pörtner OH: **Physiological and metabolic responses to hypoxia in invertebrates**, Rev. Physiol. Biochem. Pharmacol 1994, **125**: 43–147.
5. Ernest S Chang: Stressed-Out Lobsters: **Crustacean Hyperglycemic Hormone and Stress Proteins,** Bodega Marine Laboratory, University of California–Davis PO Box 247, Bodega Bay, California. 94923
6. Sousa WP: **Disturbance in marine intertidal boulder fields: the non equilibrium maintenance of species diversity**. Ecology 1979.
7. Grossman GD, Ratajczak Jr, RE, Crawford MF, Freeman MC: **Assemblage organization in stream fishes: effects of environmental variation and inter specific interactions.** Ecol. Monogr. 1998.
8. Sousa WP : **The role of disturbance in natural communities**. Annu. Rev. Ecol. Syst. 1984, **15**, 353–391.
9. Connell JH, Hughes TP, Wallace CC: **A 30-year study of coral abundance, recruitment, and disturbance at several scales in space and time**. Ecol. Monogr. 1997.
10. Huston M: **A general hypothesis of species diversity.** Am. Nat. 1979: 1131 , 81–101.
11. Huston MA:. **Biological Diversity: The Coexistence of Species on Changing Landscapes.** 1994**, C**ambridge Univ. Press, New York.
12. Menge BA : **Predation intensity in a rocky intertidal community: effect of an algal canopy, wave action and desiccation on predator feeding rates**. Ecologia 1978.

13. Witman JD, Grange KR: **Links between rain, salinity and predation in a rocky subtidal community.** Ecology 1998.

14. Sagasti A, Schaffner CL: **Effects of periodic hypoxia on mortality, feeding and predation in an estuarine epifaunal community .**Emmett Duffy School of Marine Science, Virginia Institute of Marine Science, The College of William and Mary, Gloucester Point, VA 23062-1346, USA Received 20 July 2000; received in revised form 8 February 2001; accepted 9 February 2001.

15. Tyson RV, Pearson TH: **Mod ern and ancient continental shelf anoxia: an overview**. In: Tyson RV, Pearson TH, Eds , Modern and Ancient Continental Shelf Anoxia, 58, Geological Society Special . Publication, London, 1991, 1–26.

16. Diaz RJ and Rosenberg R: **Marine benthic hypoxia: a review of its ecological effects and the behavioral responses of benthic macrofauna.** Oceanogr. Mar. Biol. Annu. Rev. 1995, 33, 245–303.

17. Sagasti A, Schaffner CL: **Effects of periodic hypoxia on mortality, feeding and predation in an estuarine epifaunal community**. Emmett Duffy School of Marine Science, Virginia Institute of Marine Science, The College of William and Mary, Gloucester Point, VA 23062-1346, USA Received 20 July 2000; received in revised form 8 February 2001; accepted 9 February 2001.

18. Diaz RJ and Rosenberg R: **Marine benthic hypoxia: a review of its ecological effects and the behavioral responses of benthic macrofauna.** Oceanogr. Mar. Biol. Annu. Rev. 1995, 33, 245–303.

19. Jørgensen BB: **Seasonal oxygen depletion in the bottom waters of a Danish fjord and its effects on the benthic community**. Oikos 1980.

20. Stachowitsch M: **Mass mortality in the Gulf of Trieste: the course of community destruction.** P. S. Z. N. I. Mar. Ecol. 1984.

21. Breitburg LD, Paerls and Farwell. **The Impact of Episodic Hypoxia on Blue Crabs (Callinectes Sapidus): From Molecules to Populations**. North Carolina State University, 2008

22. Josefson AB and Widbom B: **Differential response of benthic macrofauna and meiofauna to hypoxia in Gullmar Fjord basin.** Mar. Biol. 1988.

23. Llanso RJ: **Effects of hypoxia on estuarine benthos: the lower Rappahannock River Chesapeake Bay-a case study.** Estuarine Coastal Shelf Sci. 1992.

24. Dauer DM., Rodi AJ and Ranasinghe JA: **Effects of low dissolved oxygen events on the macrobenthos of the lower Chesapeake Bay.** Estuaries 1992.

25. Sagasti A and Schaffner CL: **Effects of periodic hypoxia on mortality, feeding and predation in an estuarine epifaunal community**. Emmett Duffy School of Marine Science, Virginia Institute of Marine Science, The College of William and Mary, Gloucester Point, VA 23062-1346, USA Received 20 July 2000; received in revised form 8 February 2001; accepted 9 February 2001.

26. Mangum C and van Winkle, W: **Responses of aquatic invertebrates to declining oxygen conditions.** Am. Zool. 1973.

27. Diaz RJ and Rosenberg R: **Marine benthic hypoxia: a review of its ecological effects and the behavioral responses of benthic macrofauna.** Oceanogr. Mar. Biol. Annu. Rev. 1995, 33, 245–303.

28. Telford M: **The effects of stress on blood sugar composition of the lobster, Homarus americanus.** Can. J. Zool. 1968.

29. Webster SG: **Measurement of crustacean hyperglycemic hormone levels inthe edible crab Cancer pagurus during emersion stress**. J. Exp. Biol. 1996.

30. Bo¨cking D, Dircksen H, and Keller R: **The crustacean neuropeptides of the CHH/MIH/GIH family: Structures and biological activities**. In K. Wiese (ed.), The crustacean nervous system 2002, 84–97. Springer, Berlin.

31. Aliko V, Faggio C, Hajdaraj G, Caci A: **Copper induced lysosomal membrane destabilisation in haemolymph cells of crab (Carcinus aestuarii, nardo, 1847) from the Narta Lagoon (Albania).** Brazilian Archives of Biology and Technology 2005, IF 0.45.

32. Qyli M. and Aliko V: **The toxic effects of chloroform stress exposure on the mediterranean green crab Carcinus aestuarii**. Albanian Journal of Agricultural Sciences. 2016, 15 (4): 177-182. ISSN: 2218

33. Martinez-G´omez C, Benedicto J, Campiolla AJ and Moore M : **Application and evaluation of**

the neutral red retention (NRR) assay for lysosomal stability in mussel populations along the Iberian Mediterranean coast. Journal of Environmental Monitoring 2008, 10, 490-499.

34. Lowe MD, Fossato UV and Depledge HM: **Contaminant induced lysosomal membrane damage in blood cells of mussels Mytilus galloprovincialis from Venice Lagoon: an in vitro study.** Marine Ecology Progress Series 1995, 129:189-196.
35. Kleinholz LH. and Little CB. **Studies on the regulation of blood sugar in Crustaceans. I. Normal values and eksperimental hyperglycemia in Libinia emarginata**. Biological Bulletin 1949.
36. Abramowitz A, Hisaw FL, Papandrea DN. **The occurrence of a diabetogenic factor in the eyestalk of crustaceans.** Biol. Bull. 1944.
37. Mortimore, G.E., Huston,J.N., and Surmacz AC:. **Quantitative correlation between proteolysis and macro- and microautophagy in mouse hepatocytes during starvation and refeedin.** Proc. Natl. Acad. Sci. USA. 1983, 80: 2179-2183.
38. Marzella L, Ahlberg J, and Glaumann H:. **An Overview of Autophagy: Morphology, Mechanism, and Regulation.** Exp. Cell Res. 1980, 129: 460-466.
39. Mortimore, G.E., Huston,J.N., and Surmacz AC:. **Quantitative correlation between proteolysis and macro- and microautophagy in mouse hepatocytes during starvation and refeedin.** Proc. Natl. Acad. Sci. USA. 1983, 80: 2179-2183.
40. Marzella L, Ahlberg J, and Glaumann,H**. An Overview of Autophagy: Morphology, Mechanism, and Regulation.** Exp. Cell Res.1980, 129: 460-466.
41. Pfeifer U. **In Lysosomes**: **Their Role in Protein Breakdown**. Glauman H and Ballard JF, Eds; 1987, 3-59. Academic Press, New York, San Francisco, London.
42. Hawkins HK: **In Pathobiology of Cell Membranes.** Trump FB and Arstila VA, Eds; 1980, 2: 252-285. Academic Press, New York, San Francisco, London.
43. Szego CM and Pietras JR: **Lysosomal functions in cellular activation: propagation of the action of the hormones and other effects**. Int. Rev. Cytol. 1984, 88: 1-302.
44. Hawkins HK: **In Pathobiology of Cell Membranes**. Trump FB and Arstila VA, Eds. 1980, 2: 252-285. Academic Press, New York, San Francisco, London.
45. Moore MN. **Cellular-responses to pollutants**. Mar. Pollut. Bull. 16: 134-139,
46. Moore MN: **Cytochemical studies on the stability of the lysosomal membrane in relation to cell function using molluscan digestive cells as a model system**. Plymouth Marine Laboratory, Prospect Place, Plymouth, PL1 3DH, UK.
47. Moore MN: **Cytochemical demonstration of latency of lysosomal hydrolases in digestive cells of the common mussel, Mytilus edulis, and changes induced by thermal stress**. Cell Tiss. Res. 1976.
48. Moore MN, Lowe MD and Fieth MEP:. **Reponses of lysosomes in the digestive cells of the common mussels, Mytilus edulis, to sex steroids and cortisol.** Cell Tiss. Res. 1978.