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Variations in Subcuticular Acid Phosphatase Activity During the Molting Cycle of the Euphausiid Crustacean, Thysanoessa raschii *(M. Sars) Hansen*[1]

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ABSTRACT

A histochemical technique, applicable to intact euphausiids, has been used in the laboratory to demonstrate variations in the level of subcuticular acid phosphatase activity during the molting cycle of *Thysanoessa raschii*; it shows that there is a marked increase in such activity during the 24 hours in which ecdysis occurs. We suggest that the technique could form a useful tool in future studies on euphausiid biology since it would allow, for example, assessments of the numbers of individuals in any one collection or population that is passing through this particular 24-hour period at the time of sampling.

Introduction. Most of the early investigations into the molting cycle of crustaceans (reviewed by Passano 1960) were physiological in nature and were concerned primarily with various functional aspects of the process in relation to the particular molting individual. Most of these earlier studies were carried out on the Decapoda, especially brachyurans, and knowledge of the molting cycles of other orders is relatively scanty.

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In recent years, however, the molting cycle of crustaceans has attracted the attention of ecologists, fisheries workers, and oceanographers, and studies by them have been centered on crustaceans that form significant components of the food web of the oceans. The focus of attention in this context has been upon entire populations rather than on individual members of any one species. The Euphausiacea are among the most important components of the marine food web, and molting frequency and related topics in this order are now being studied in some detail. In *Euphausia pacifica*, for example, Lasker (1964, 1966) has shown that the molting interval is 5 days, with a standard deviation of 1 day (range 4–7 days), and that each cast molt averages 10% of the animal's dry weight. Thus each euphausiid contributes its full dry weight of material to the ocean every 50 days. When the enormous size of the euphausiid population is considered, this is a significant contribution of synthesized material to the detritus of the ocean. Similar data have been recorded for *E. eximia, Nyctiphanes simplex*, and *Thysanoessa spinifera* (Jerde and Lasker 1966).

Laboratory observations such as those by Lasker can be used to establish molting frequency in any species that can tolerate laboratory conditions. So far, however, no method has been available that permits detection of molting frequency on shipboard in samples from routine collections except by searching the samples for the proportion of cast molts present.

In the course of histochemical studies on enzymic activity in the alimentary and nervous systems of various species of copepods (using whole-mount techniques as described by Halton and Jennings 1964, Gibson and Jennings 1967), Jennings, Loftus, and Parrish (unpublished data) noted a generalized subcuticular reaction in a small proportion of the naupliar stages. This was interpreted as possible lysosomal phosphatase activity concerned with molting, but the hypothesis could not be tested satisfactorily on the copepod material. Availability of laboratory stocks of the euphausiid *Thysanoessa raschii*, whose molting cycle was under study by one of us (L. G. H.) and whose molting frequency was therefore known, made possible a similar study of subcuticular acid phosphatase activity in this species. Thus an attempt was made to relate such activity more specifically to the molting cycle.

Furthermore, it was hoped that such a study might lead to the development of a technique for assessment of the proportion of molting individuals in field samples of euphausiid populations. The need for, and the potential usefulness of, such a technique had been pointed out to us by Peter Wiebe of the Woods Hole Oceanographic Institution.

Methods. Specimens of *Thysanoessa raschii* (M. Sars) Hansen were collected in surface-to-bottom oblique tows (using a 75-cm net, mesh size 1.024 cm) in the Gulf of Maine off Provincetown, Massachusetts, during July and early August 1970; the bottom depth was 61 m. The net was towed at depth for 5 minutes, with approximately 4 minutes allowed for recovery time.

After recovery, the cod end was immersed in a polyethylene tub filled with seawater taken from the surface at the tow site. Due to the large mesh size of the net, relatively few animals other than euphausiids were taken, and this facilitated sorting of the collection and avoided prolonged exposure of the animals to sunlight. Euphausiids caught in the net were discarded, since Conover (1966) has pointed out that such animals are probably injured during the tow. Free-swimming individuals were transferred in plastic spoons to polystyrene pint containers filled with seawater. The sealed containers, with 3–4 specimens per container, were kept in an ice chest for the 5-hour return trip to the laboratory. The water was aerated after 2.5 hours, and dead euphausiids were removed. Mortality during this travel time was very low.

In the laboratory the euphausiids were maintained individually in polystyrene containers containing 350 ml of filtered seawater (taken at the collection site). The organisms were kept in the dark at 10°C. Necessary maintenance work was done with the aid of only a flashlight, and even this small amount of illumination appeared to activate the animals and to increase their swimming. This supported the observation by Mackintosh (1967) that *Euphausia superba* was easier to maintain when kept in total darkness.

The seawater was changed every three days. Immediately after each change, 300–400 *Artemia* nauplii and 10 ml of a mixture of *Isochrysis galbani*, *Monochrysis lutheri*, *Phaeodactylum tricornutum*, and *Cyclotella* clone 3H were added as food. The euphausiids were handled with a wide-bore pipette (3 cm I. D.) but handling was kept to a minimum.

Checks made twice daily showed that molting generally occurs between 2400 and 0800 hours. The molting frequency for each individual was established, and in subsequent studies on variations in subcuticular acid phosphatase activity during the molting cycle, the cycle was timed from the last 2400-hour check before a cast molt was found. It proved impracticable to establish the precise time of molting for each individual; thus all timings within the molting cycle were approximate only, in the sense that molting could have occurred at any time during the first 8 hours of the cycle.

To study variation in the subcuticular acid phophatase activity throughout the molting cycle, euphausiids were fixed at 12-hour intervals and then processed in toto for the demonstration of acid phosphatase activity by using a slight modification of the azo dye method of Burstone (1958).

Fixation was for 12 hours at 1°C in 10% formalin, buffered to pH 7.0 and prepared from: 10 ml of formalin, commercial, 40% w/v HCHO; 90 ml of distilled water; 0.4 g of sodium phosphate monobasic $NaH_2PO_4 . H_2O$; and 0.6 g of sodium phosphate dibasic $Na_2HPO_4 . 7H_2O$. Specimens were rinsed in distilled water, passed through 50%, 100%, and 50% acetone for 5 minutes at each grade, and then returned to distilled water for a final rinse before incubation. All solutions were held at 1°C. Incubation was for 8 hours at 20°C, in a freshly prepared mixture of: 4 mg of naphthol AS-BI phosphate, dissolved in

0.25 ml of dimethyl formamide, 25 ml of 0.2 M acetate buffer (pH 5.2), 2 drops of 10% manganese chloride, and 35 mg fast red violet LB salt. After incubation, specimens were rinsed in distilled water, briefly (5–10 seconds) washed in dimethyl formamide, rinsed again in distilled water, and then mounted on depression slides in glycerol jelly. Sites of acid phosphatase activity in the specimens appeared red to reddish purple.

In control experiments, the enzymic nature of the histochemical reaction was confirmed by holding the euphausiids at 90°C for 5 minutes immediately before incubation in the standard medium and by incubating others treated in the normal manner in the standard medium from which the specific substrate, naphthol AS-BI phosphate, was omitted.

The preincubation acetone rinses were necessary to remove lipid deposits. Both the fast red violet LB salt and the red reaction product are soluble to some extent in lipid; the resultant diffuse coloration prohibits localization of the sites of enzymic activity. The dimethyl formamide rinse after incubation removes the crystals of naphthol AS-BI phosphate that may form on the appendages and general body surface; but this rinse must be as brief as possible to prevent removal of the colored reaction product.

Results. The specimens of *Thysanoessa raschii*, taken from a single site in two collections separated by seven days, fell into three groups as regards molting frequency in the laboratory (Table I). Group B, with a molting cycle of 96 hours, was used for most of the experiments carried out during the development of this technique. A smaller number of experiments were done with Group A animals, which molted at 72 hours; one animal from Group C (120-hour intervals) was also tested.

A positive reaction to this technique for the demonstration of subcuticular acid phosphatase activity consists of a diffuse red to reddish-purple coloration beneath the cuticle over most of the body, but especially in the telson, in the thoracic and cephalic appendages, and in the dorsal region of the carapace. All animals tested, irrespective of their stage of development, gave this basic reaction at the time of fixation; presumably this reaction represents the normal background level of enzymic activity. However, the intensity of the reaction, i.e., the depth of coloration reached within the standard incubation period of 8 hours at 20°C, increased significantly as acdysis (used in this context to indicate actual exuviation of the old cuticle) was approached.

Table I. Molting intervals of *T. raschii*.

Group	Number	Molt intervals* (hours)
A	21	72
B	60	96
C	19	120

*Hours between two consecutive ecdyses.

All *T. raschii*, fixed 24, 36, 48, and 60 hours after the last check prior to a known molting, showed the same diffuse and rather weak background reaction. At the 72-hour stage, the coloration was

stronger, and the peak intensity was reached in the 84-hour specimens, with most, or all, of the body being a deep reddish purple. This coloration was maintained in the 96-hour specimens that were fixed, presumably within 8 hours of complete ecdysis.

One somewhat unexpected result was the continuation of the high level of subcuticular phosphatase activity through actual acdysis and its appearance in the 12-hour stage. Presumably this reaction, which fell to the normal background level within the next 12 hours, reflects continued high-level metabolic activities in association with the newly exposed cuticle, comparable to the water absorption and mineralization that occurs in the cuticle of newly molted brachyurans (Drach 1939, Passano 1960).

Table II. Variations in the intensity of the histochemical reaction for subcuticular acid phosphatase activity in *T. raschii* of Group B during the 96-hour molting cycle. An average of five specimens per time interval was examined. See *Methods* regarding time.

Time from last check before molting (hours)	Subcuticular reaction*
12	+++
24	+
36	+
48	+
60	+
72	++
84	+++
96	+++

* + = normal background reaction.
++ = medium reaction, approximately twice the normal strength.
+++ = intense reaction; most or all of animal deeply colored.

T. raschii (Group A), molting every 72 hours, gave similar results when tested but, as would be expected, the subcuticular reaction started to increase in intensity at the 48-hour stage in the molting cycle and showed a peak intensity at 60 and 72 hours.

A single specimen from Group C, molting at 120 hours, was fixed at 114 hours and showed an intensive positive reaction when incubated.

Control experiments, using both heat-treated *T. raschii* incubated in a normal medium and normal *T. raschii* incubated in the absence of the specific substrate, gave completely negative results. The euphausiids remained creamy white and showed no trace of the reddish reaction product.

Since it was hoped that this technique might prove to be of use on shipboard during routine sampling of euphausiid populations, attempts were made to simplify the procedure by substituting acetone as the fixative. In addition to eliminating one reagent, it was thought that the fixation time could be reduced from 12 hours, since acetone penetrates and precipitates animal tissues much more rapidly than formalin. Unfortunately fixation in acetone without pretreatment in buffered formalin reduces the intensity of the final coloration to about one third of the expected amount, consequently the results are less easy to interpret. The loss in intensity of the reaction may be due to direct inhibition of enzymic activity, or, to a hindrance of the inward diffusion of the reagent

caused by some action of the acetone on the crustacean's cuticle. This action is prevented in some way by prefixation in buffered formalin.

Discussion. Our results demonstrate that there is a direct relationship between the intensity of the histochemical reaction for subcuticular acid phosphatase activity, as visualized in preparations of entire euphausiids, and the stage reached in the molting cycle. While precise timing of ecdysis has not been achieved, it is possible to state that the development of an intense reddish-purple coloration in euphausiids subjected to this technique shows that the crustacean is somewhere within the 24 hours of the molting cycle that include ecdysis. It is impossible to determine, on this criterion alone, whether the animal is entering the last 12 hours of the molting cycle or whether it has in fact molted and is entering the intermolting period. Texture of the cuticle cannot be used as an additional criterion, since it is considerably altered by the treatment with formalin and acetone. No significant differences could be found in the cuticle of immediate premolt and postmolt individuals after such treatment.

Biochemical and histological studies of the epidermis during the comparable period in the molting cycles of brachyurans (summarized by Passano 1960) have shown particularly that the glycogen content changes and that the epidermal cells enlarge while their oxygen consumption rises. The enzymic activity visualized in *T. raschii*, using the present technique, is clearly a manifestation of similar increased metabolic activity.

This relationship of the intensity of the histochemical reaction (in preparations of entire euphausiids) to the particular stage reached in the molting cycle appears to be valid, irrespective of the total length of the cycle; comparable results were obtained in *T. raschii* with cycles of 72, 96, and 120 hours. The technique is reported here in the hope that it may prove a useful tool in future studies on the biology of euphausiids and on their role in the food web of the ocean. For example, it should facilitate further studies on the molting cycle by allowing assessments to be made of the numbers of individuals in any one collection or population that, at the time of sampling, are passing through the 24-hour period in which ecdysis occurs. Jennings, Loftus, and Parrish (unpublished data) noted identical reactions in naupliar stages of copepods, and it seems likely, therefore, that the technique may be applicable to any planktonic crustacean that possesses a clear cuticle.

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