AN ABSTRACT OF THE THESIS OF

Julie Ellen Dunn for the degree of Master of Science

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Title: Development of a Marine Bioassay System Using the Gooseneck Barnacle, Pollicipes polymerus

Abstract Approved: Redacted for privacy

Michael C. Mix

The purpose of this study was to develop a marine bioassay procedure using eggs and larvae of Pollicipes polymerus, a stalked barnacle common on the west coast of North America.

A series of experiments were run to determine optimal culture conditions for the eggs, to see if they could be grown without antibiotics, and to generate the necessary data on development time, hatching, and molting success required to design future experiments.

Next, two bioassays were run to determine how the system performed in actual use. A phthalate ester plasticizer, dibutyl phthalate (DBP), was used as the test compound. The first of these experiments indicated there was a significant drop in molting success from larval stage I to stage II when eggs were exposed to 1000 ppb DBP. Variances in molting success, sometimes high in previous experiments, became unacceptable in this one, so the next experiment was designed to reduce these variances by more accurately identifying the time of first egg hatching.

The end point used in previous experiments (3 days after first hatching) proved to be insufficient for enough of the controls to molt and, therefore, no conclusions could be drawn on the effects of DBP. The experiment did indicate how excessive variance in molting success could be avoided in future experiments, and how overall molting success could be improved. Hatching successes obtained in all these experiments compared favorably to those obtained by previous investigators.

While testing the synthetic seawater used in the experiments for background phthalate levels, it was found that several phthalates, particularly DBP and diethylhexyl phthalate (DEHP) were persistent contaminants. Dibutyl phthalate levels could be reduced by treatment with activated charcoal.

Tests were conducted to determine how much DBP remained in solution between water changes. Dibutyl phthalate loss was found to increase with increasing initial DBP concentration, but DBP loss from this system over 24 hours compared favorably to that observed in a similar experiment employing larval marine crustaceans.

A design for equipment to culture P. polymerus in vitro and suggestions for conducting bioassay studies with eggs and larvae are described.

Development of a Marine Bioassay System Using the Gooseneck Barnacle, *Pollicipes polymerus*

by

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS AND DEFINITIONS

ANOVA - Analysis of variance

DBP - Dibutyl phthalate

DEP - Diethyl phthalate

DEHP - Diethylhexyl phthalate

DOP - Dioctyl phthalate

HPLC - High performance liquid chromatography

Hatching Index =

$$\frac{\text{no. stage I larvae + no. post-stage I larvae}}{\text{no. stage I larvae + no. post-stage I larvae + no. unhatched eggs}} \times 100$$

Molting Index =

$$\frac{\text{no. post-stage I larvae}}{\text{no. stage I larvae + no. post-stage I larvae}} \times 100$$

Development of a Marine Bioassay System Using the Gooseneck Barnacle, Pollicipes polymerus

INTRODUCTION

The term "bioassay" has had several meanings over the years. Originally, it referred to the practice of using living organisms to detect small quantities of toxicants, the quantity of toxicant being indicated by the number of organisms killed in a given amount of time (Tinsley 1979). With the introduction of modern analytical techniques that practice became obsolete and the term came to refer to tests using organisms to test the potency of drugs; more recently it has come to encompass a wide variety of toxicity tests (APHA, 1976). The new generation of bioassay tests have become valuable tools in water quality studies as increasing numbers of new chemicals, whose effects upon organisms are poorly understood, are synthesized.

Different types of toxicity bioassays are referred to by a variety of terms and do not always have consistent definitions; the following are working definitions used in this study. Acute bioassays are short-term tests (measured in hours or a few days) most often used to measure lethal doses or concentrations. Chronic bioassays are medium and long-term tests which usually test lower concentrations of toxicants and examine sub-lethal effects or lethal effects which may not become evident until a certain part of the life cycle (such as reproduction) or until the organism has been exposed to the chemical for a longer time. Chronic bioassays encompassing the complete life cycle of a test organism provide the most

information but they can be time consuming and expensive. Partial life cycle tests employing the most sensitive stages of the life cycle can be a cost-effective alternative.

In order to set meaningful water quality standards there is a need to know how a substance will affect a representative spectrum of organisms in an ecosystem; unfortunately, that is often not the case when standards are written. Invertebrates play a crucial role in marine ecosystems at all trophic levels, yet, they are under-represented in existing bioassay techniques (Maciorowski and Clarke 1980). Of those for which standardized procedures are available, a number of phyla are absent and others are heavily biased toward only a few subclasses. For example, all of the marine and brackish water crustaceans suggested as test species in Standard Methods for the Examination of Water and Wastewater (1976) are malacostracans or copepods. Reasons for this include the lack of culture methods for most invertebrate species and a preference for using commercially important species.

Although the lack of methodology is exacerbated by a paucity of knowledge about the life histories and habits of many invertebrates, these animals possess attributes which make them potentially useful in bioassays. They are often small and have comparatively short life cycles so that long-term tests can be run less expensively than with vertebrates. Also, a number of the larger, longer-lived species have sensitive developmental and juvenile stages which are small, easy to work with, and otherwise ideal for partial life cycle tests (Maciorowski and Clarke 1980). One species with these characteristics

is the gooseneck barnacle, Pollicipes polymerus.

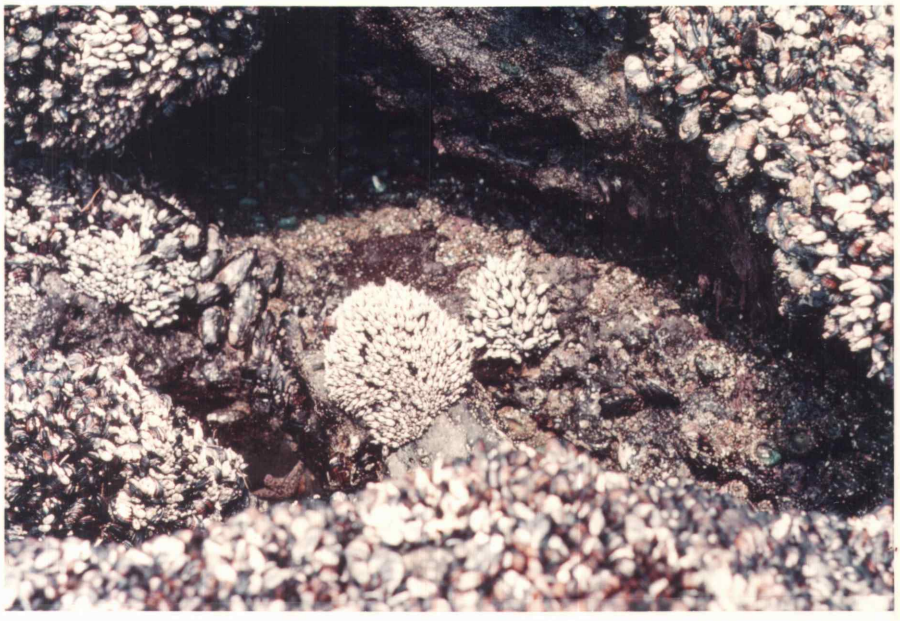
Pollicipes polymerus

Pollicipes polymerus is a common intertidal invertebrate along the west coast of North America, ranging from British Columbia to Baja California (Cimberg 1981). It is often found with the mussel, Mytilus californianus, and the starfish, Pisaster ochraceous (Figs. 1, 2a and b). Adults feed on detritus and small crustaceans such as amphipods, while the free-swimming larvae are planktivores; thus, P. polymerus occupies a unique position in the rocky intertidal food web (Fig. 3) (Hedgpeth, Ricketts, and Calvin 1973).

P. polymerus eggs and larvae have been cultured in vitro from fertilization through settlement (Lewis 1975a, b) and different culture methods have been developed (Lewis 1975a, Mix, Bunting, and Abbott 1979). P. polymerus larvae have been used in studies involving low-level tritium exposure (Abbott 1975, Abbott and Mix 1979) and larvae of other barnacle species have been used in acute (Christie and Crisp 1966, Morton and Wu 1977, Blundo 1978) and chronic (Rosenberg 1972) toxicity tests.

Spawning by the hermaphroditic adults occurs when water reaches an optimum temperature; 14° C for the subspecies existing north of Point Conception, CA (Cimberg 1981). In Oregon the spawning season can be induced in the laboratory by gradually raising the water temperature to 14° C (Cimberg 1981) and eggs and larvae could be available throughout the year.

Figure 1. Oregon rocky intertidal area; habitat of P. polymerus. Blue-black color of rocks is from heavy cover of Mytilus.



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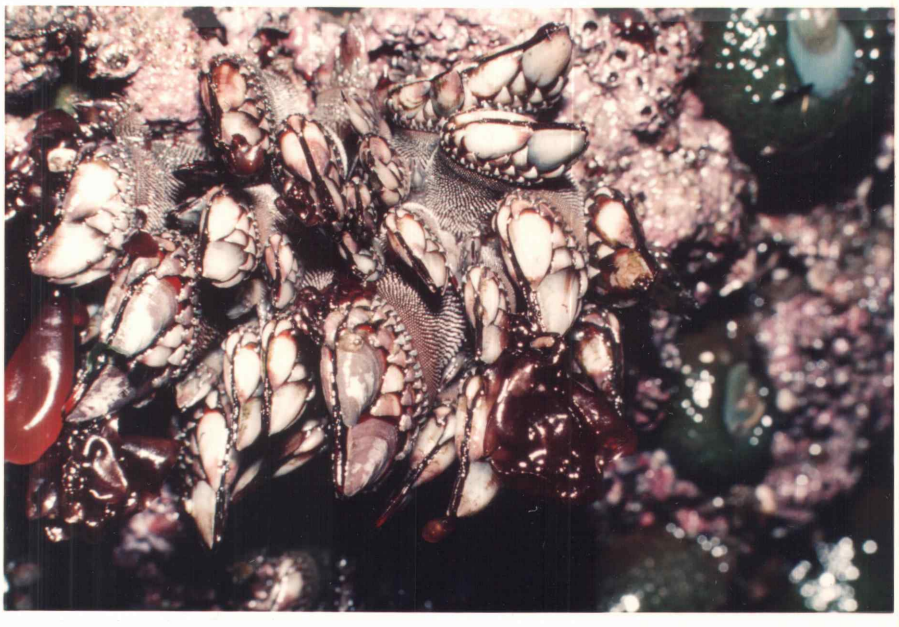

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Figure 2. Colonies of P. polymerus. Adults appear chalky white in exposed area (a above) but those in protected, shady areas often have pearly white plates with some purple or brown coloration (b above).

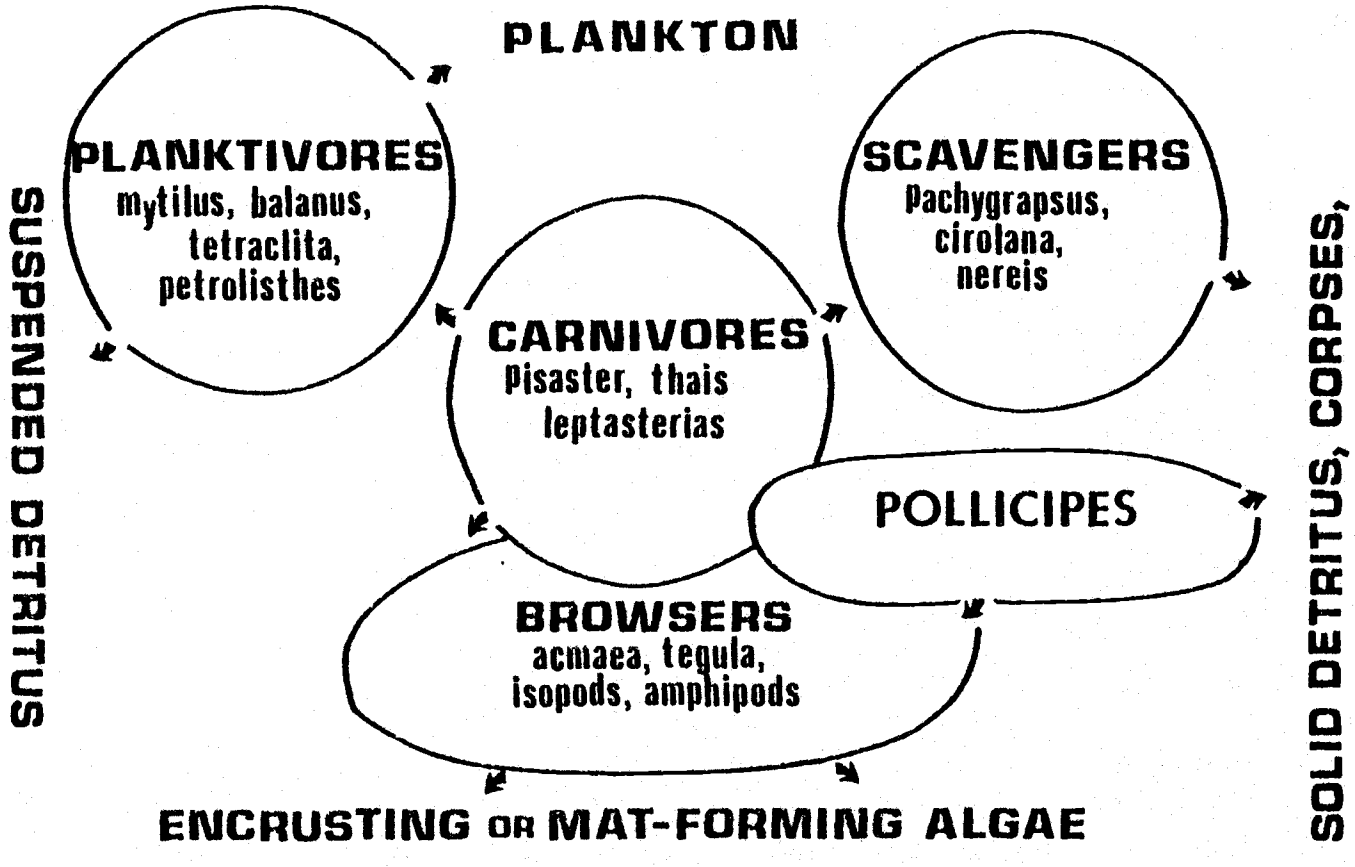


Figure 3. Simplified food web of West Coast mussel bed (after Hedgpeth, Ricketts and Calvin 1973).

Fertilized eggs are extruded into two ovigerous lamellae (egg masses) which remain inside the mantle cavity until hatching occurs (Smith and Carlton 1975) (Fig. 4). The approximate age of the egg masses can be determined by color and texture. Newly fertilized egg masses are pale white-orange and flimsy. After formation of the blastoderm until formation of the naupliar segments, they are light orange and brittle. In later stages they are a bright, deep orange and finally turn brown and crumble as hatching occurs (Fig. 5). At 14° C, the time from fertilization to hatching averages 25 days (Lewis 1975b).

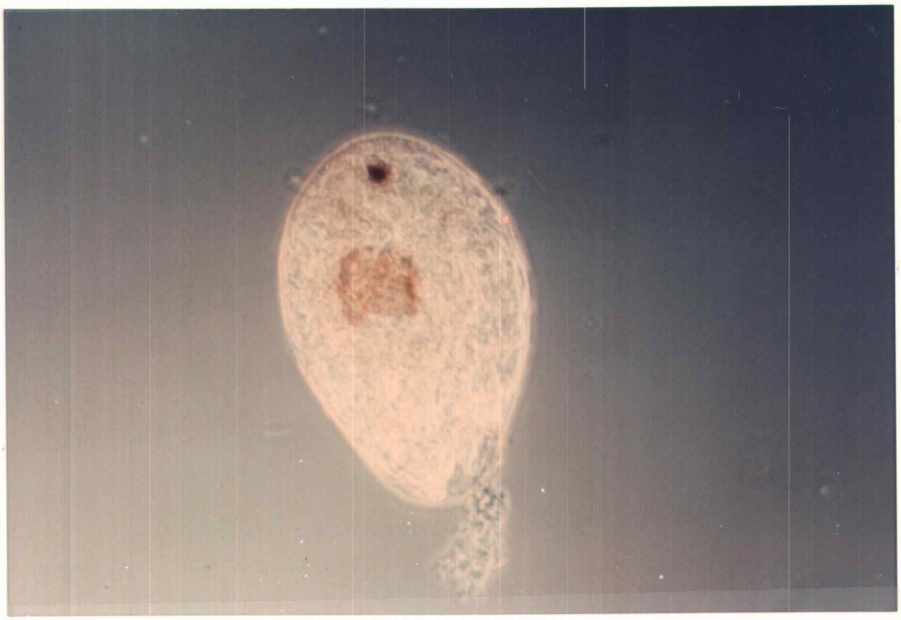
Hatching is followed by six free-swimming naupliar stages. Stage I nauplii (Fig. 6) are morphologically distinct from later naupliar stages; they do not feed and molt within hours to stage II (Fig. 7). Stages II-VI are planktotrophic and morphologically similar except for placement and angle of appendages and successively larger sizes (Fig. 8) (Lewis 1975b). Stage VI nauplii molt into cypris larvae with a well developed sensory apparatus used to locate an appropriate settling spot. When a spot is located the cyprid cements itself into place and molts into a sedentary adult (Crisp 1974, Lewis 1975b).

In summary, the eggs and larvae of P. polymerus are well suited for use in partial life cycle bioassays because of the ecological importance of the species, availability of eggs, existence of culture methods, and, these stages comprise the most sensitive part of the life cycle (Maciorowski and Clarke 1980). Other attributes of P. polymerus lend themselves to sound experimental design and

a

b

Figure 4. Egg masses as seen in mantle cavity after adult animal has been removed. a. Light orange color characteristic of early developmental stage. This egg mass may start to hatch in 2-3 weeks. b. Bright orange color characteristic of later stage. This egg mass will probably start to hatch within the next week.



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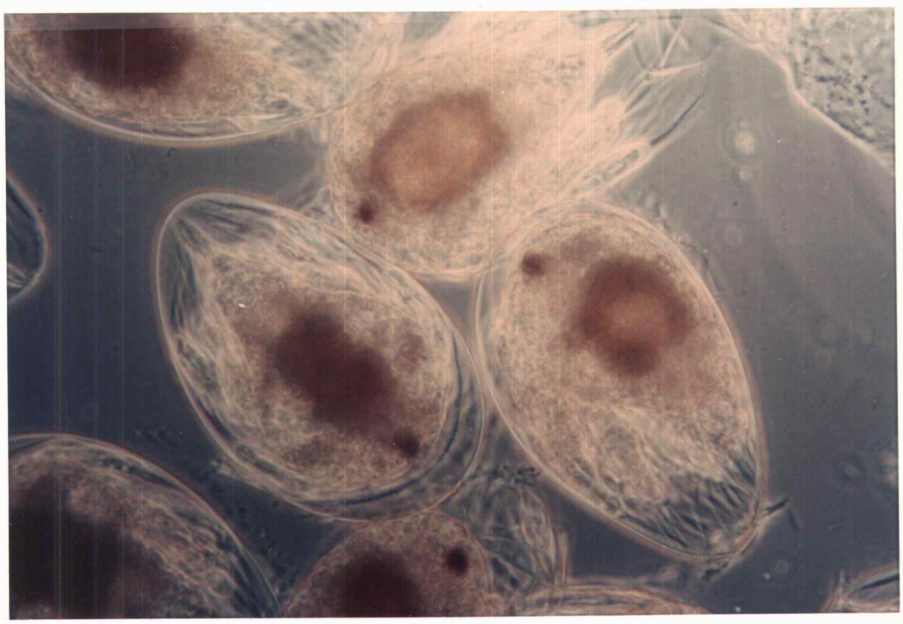
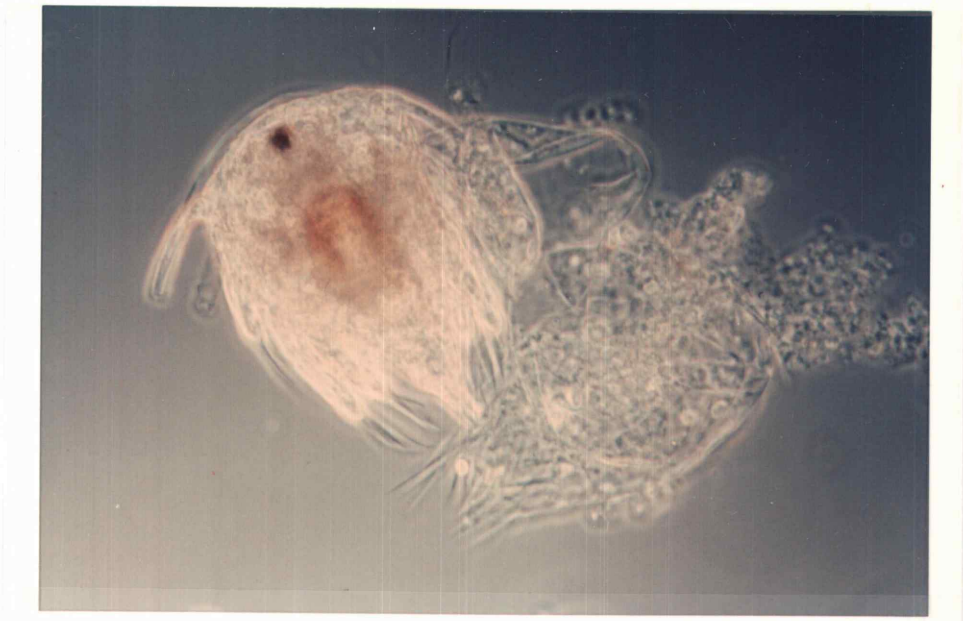


b

Figure 5. P. polymerus eggs; 250X using phase-contrast optics.
a. Later stage egg; several days prior to hatch.
b. Egg just prior to hatching. Appendages are distinctly visible; membrane has moved away from unhatched nauplius.



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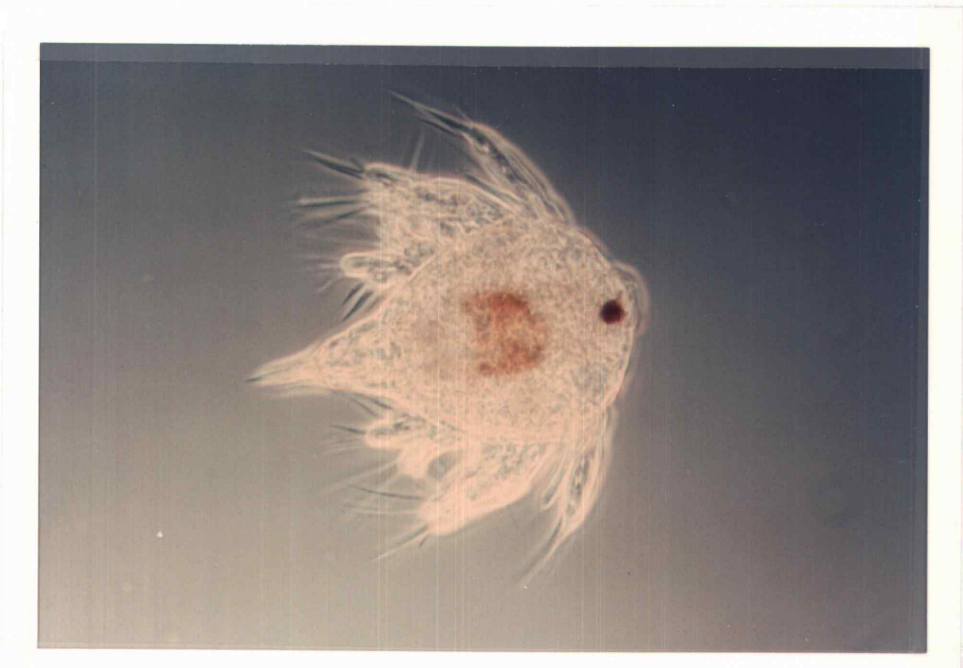

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Figure 6. Stage I nauplii; 250X, phase-contrast optics. a. In process of hatching. b. After hatching.

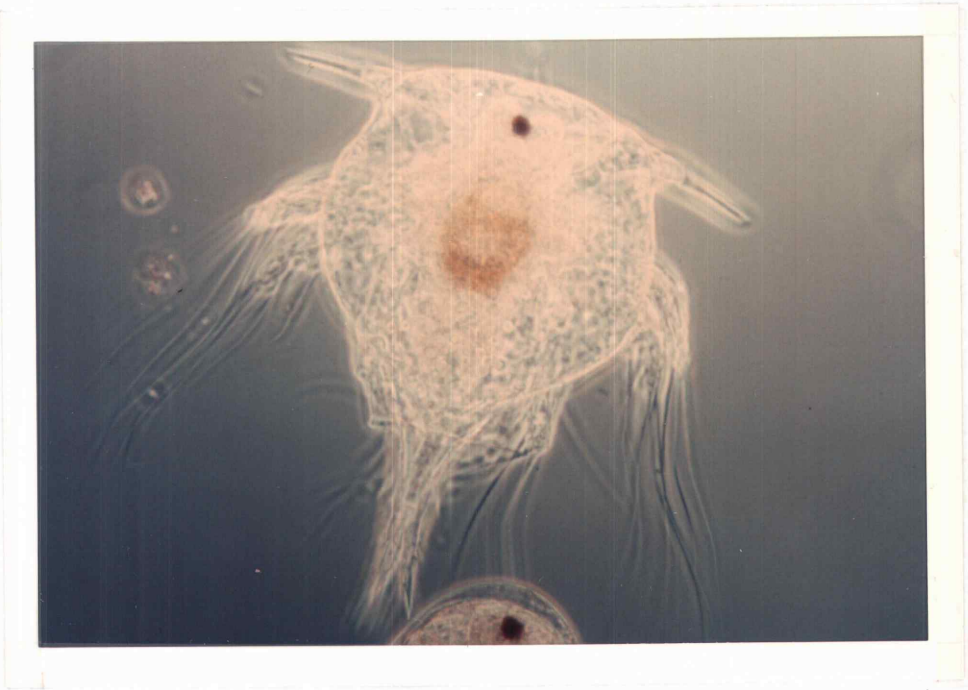
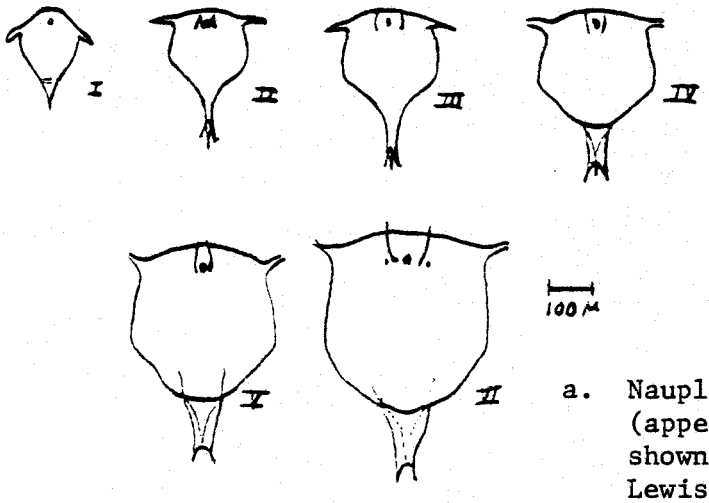
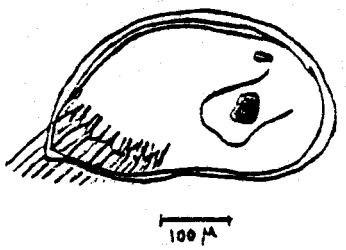


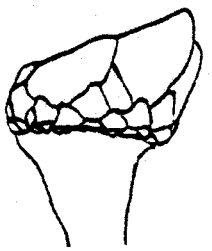
Figure 7. Stage II nauplius; 250X, phase-contrast optics. Note pronounced tail and "horns."



a. Naupliar stages (appendages not shown) after Lewis (1975b).



b. Cyprid larva, after Lewis (1975b).



c. Adult, from Smith and Carlton (1975).

Figure 8. Pollicipes life stages.

statistical manipulation. Egg masses grow well when cut into halves or quarters (Lewis 1975a, Mix et al. 1979). Since each adult produces two egg masses with over 20,000 eggs in each (Abbott 1975), eight genetically similar "units" can be obtained per adult with the number of organisms per unit limited in practice only by how large a sub-sample one desires to count.

Research Objectives

The primary goal of this study was to develop a partial life cycle bioassay procedure using the eggs and larvae of P. polymerus. First, existing culture methods and apparatus were evaluated in terms of suitability for bioassays. Factors examined and evaluated included similarity to natural conditions, reliability, ease of use, elimination of any potentially interfering elements such as toxic or adsorptive surfaces from the culture system, and identification of appropriate events and end points to use in experiments.

Next, experiments were performed to determine how well the system functioned in actual use by exposing eggs to low (10, 100 and 1000 ppb) concentrations of the plasticizer, dibutyl phthlate (DBP). The effects of DBP upon hatching success, molting success (from stage I to stage II), and the number of days from collection of eggs to first hatching were examined.

Dibutyl Phthalate

Dibutyl phthalate was selected for the test substance because it has characteristics which could make it a potentially significant

marine pollutant, yet very little work has been done to determine its effects upon sensitive marine invertebrates.

Dibutyl phthalate is one of a number of phthalate esters used to impart flexibility to plastics. They may account for up to 40% of the final weight of flexible plastics such as Tygon tubing, and are also used in a variety of other products and processes. One billion pounds of phthalate esters were manufactured in 1972 (Graham 1973) and the use of plastics has increased since that time. Since phthalates are not incorporated into the polymer they can, and do, leach out. They have been identified as widespread freshwater and marine pollutants, especially the two most frequently used esters, diethylhexyl phthalate (DEHP) and dibutyl phthalate (DBP) (Mayer, Stalling and Johnson 1972; Hites 1973; Giam, Chan and Neff 1978a; Giam, Chan, Neff and Atlas 1978b). These have been found in surface waters of the Gulf of Mexico in concentrations of about 100 ppb (DBP) and 130 ppb (DEHP) (Giam et al. 1978a).

Although of low acute toxicity (Mayer and Sanders 1973, Cassarett and Doull 1980), phthalate esters are subject to biomagnification processes similar to DDT in aquatic species. Crustaceans and molluscs are less able to rid themselves of phthalates compared to fish (Metcalf, Booth, Schuth, Hansen and Lu 1973).

DBP is more soluble in seawater than DEHP (50 ppm compared to one ppm) and is more toxic to larval crustaceans. Grass shrimp larvae exposed to 10 ppm DBP from the time of hatching show 100% mortality after three days (Laughlin, Neff, Hrung, Goodwin and

Giam 1978) and one ppm significantly decreases hatching success in brine shrimp eggs (Sugawara 1974).

MATERIALS AND METHODS

Culture Apparatus

The basic design for culture chambers and cooling apparatus was a modification of a type described by Mix et al. (1979). The modified version eliminated excessively adsorptive or potentially toxic surfaces from the culture chambers, and simplified the system to make it easier to conduct bioassay studies.

The culture chamber consisted of a 30 ml coarse filter Buchner funnel (Fig. 9a) with the stem shortened to about 3.5 cm. A 25 cm length of teflon-lined autoclavable plastic tubing (Bev-a-line V HT) (Fig. 9b) was connected to the stem. When the tubing was bent and clamped to the rack holding the culture chamber, the outlet to the chamber was closed; when the tubing was unclamped, the water in the chamber could be drained out by gravity or be gently aspirated into a suction flask.

Covers were made by drilling a hole in the center of a 5 cm watch glass and plugging the hole with a septum of silicone aquarium seal (Fig. 9c). A bead of aquarium seal, about 3 mm high, was placed 5 mm from the edge on the convex surface of the glass (Fig. 9d) to hold the cover securely in place and to discourage entry of airborne mold spores or bacteria. A length of 0.8 mm inner diameter teflon tubing with a glass wool filter (Fig. 9e) was introduced through the septum for an aeration tube; the other end was inserted directly through the wall of latex rubber tubing connected to an aquarium

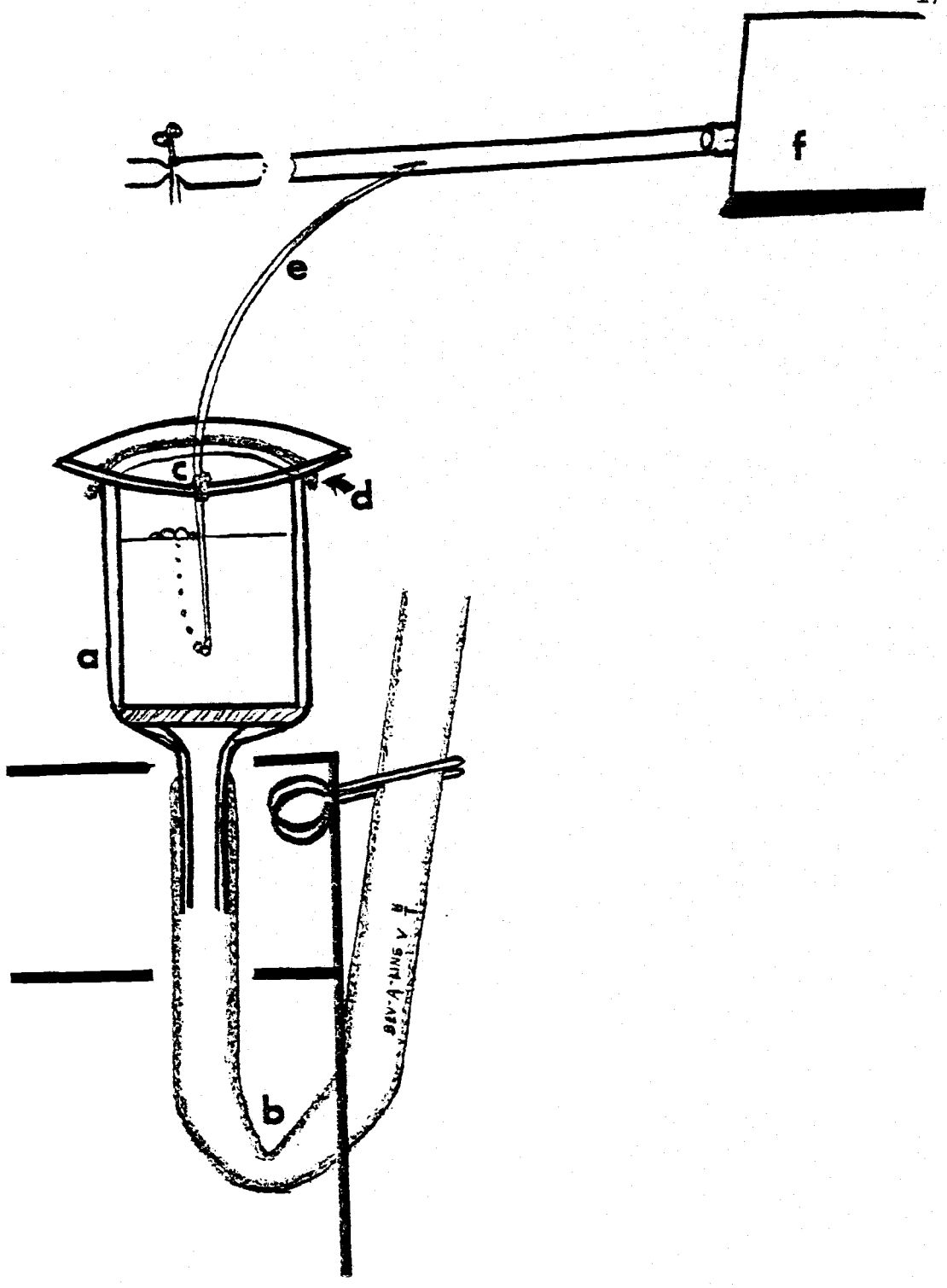


Figure 9. Culture apparatus. See text, page 16, for a complete explanation.

pump (Fig. 9f).

Cooling System

The cooling system was a 164 x 49 x 17 cm tray (Fig. 10a) draining into a 24-gallon reservoir (Fig. 10b) connected to a portable cooling unit (Fig. 10c) by a submergible pump (Fig. 10d). Racks containing culture chambers were placed directly in the tray. The thermostat on the cooling unit and the amount of culture chamber coming in contact with the cooling water were adjusted so that the water inside the chambers was maintained at 14 ± 1° C. To avoid contamination by the cooling water, the thermostat was placed on a cold setting and the chambers placed with only their bottoms under water; this treatment and constant aeration resulted in an even temperature in the chambers.

Preparation of Culture Chambers

Previous investigators (Lewis 1975a, Abbott and Mix 1979, Mix et al. 1979) had found it necessary to use antibiotics to keep the Pollicipes cultures from being overrun by contaminating micro-organisms. Since use of antibiotics in bioassays is proscribed, one goal of this study was to eliminate contamination by other means. All parts of the culture chamber coming into contact with the developing eggs or the water used in the experiment were autoclaved prior to use, and care was taken in designing and performing the experiments to avoid possible routes of contamination.

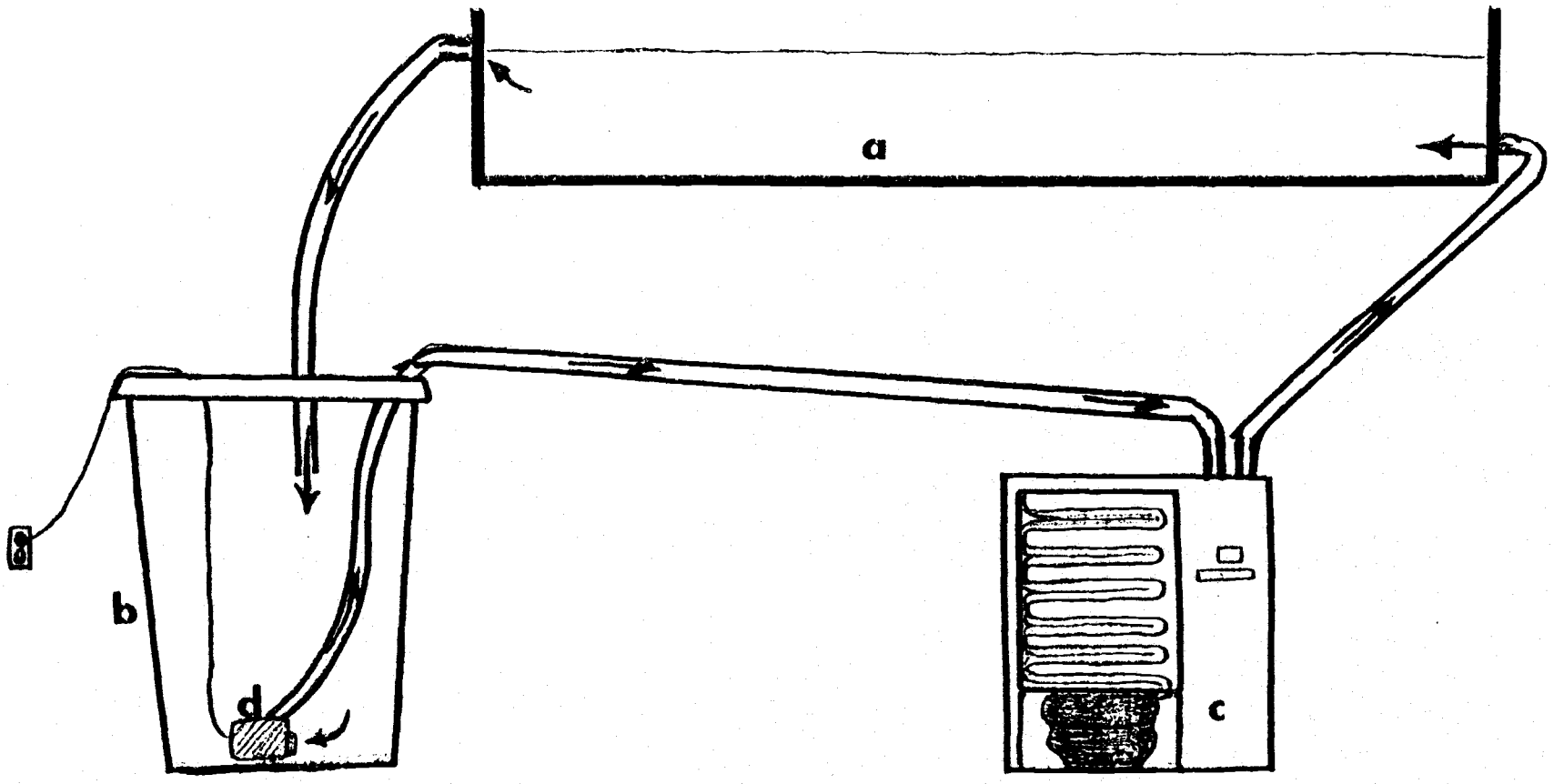


Figure 10. Cooling system. See text, page 18, for a complete explanation. Arrows indicate direction of water flow.

Preparation of Seawater

Seawater was prepared from Instant Ocean Sea Salts. Sea salts were mixed with hot tap water and allowed to cool to room temperature. The salinity was checked with a hydrometer (Zerbe and Taylor 1953) and adjusted to 29-32 ppt. The water was filtered through a pressed glass wool prefilter, then through a 0.45 µm membrane filter Fig. 11), and autoclaved (15 psi for 20 min). Salinity was checked again after autoclaving the first few batches but as the salinity never changed by more than 1 ppt, this process was terminated.

Seawater used in experiments 1-5 was prepared and autoclaved in plastic carboys. Water used in experiment 6 was prepared and autoclaved in glass bottles, and was run through a 35 x 4.5 cm column of granular activated charcoal prior to filtering.

Collection and Maintenance of Cultures

Egg masses were collected from barnacles in a colony at Yaquina Head, OR (Fig. 12). Adults were opened with a small scissors and both egg masses were removed with a teflon tape-coated forceps and placed in a 15 ml glass vial containing seawater (filtered and autoclaved as described above) and at least 2 cm of air space. Vials were sealed in plastic bags and transported back to Corvallis in a styrofoam chest filled with cold (ambient) seawater. Immediately upon arrival, egg masses were rinsed in fresh synthetic seawater, cut into quarters, and each quarter was placed in a separate culture chamber.

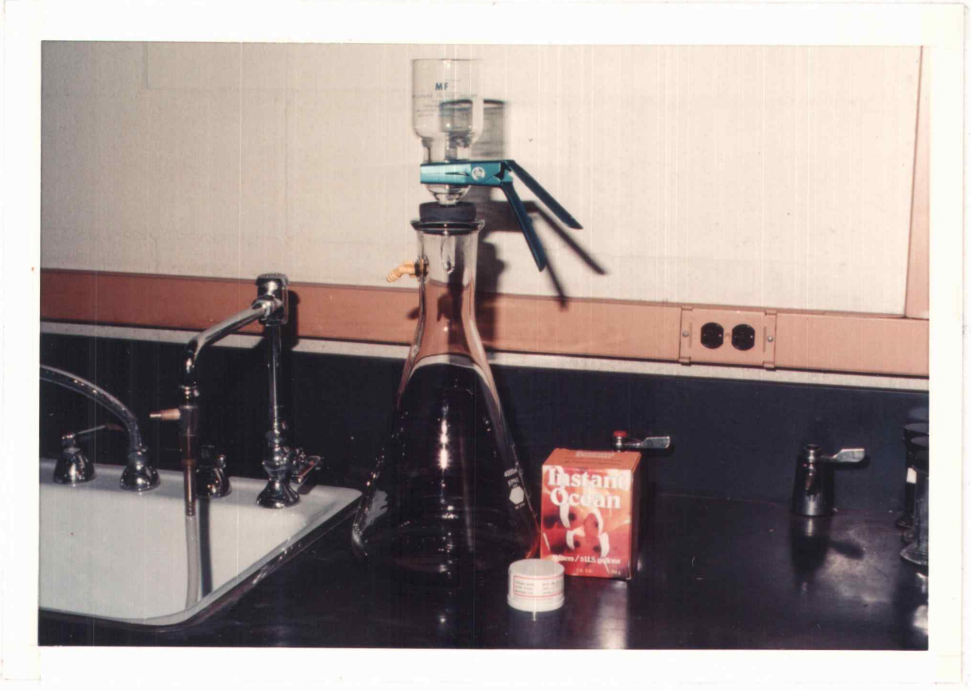


Figure 11. Filtration system used for preparation of synthetic sea salts.

Figure 12. Collection of egg mass.

Culture chambers were maintained under the lighting and temperature regimes specified for each experiment. All chambers were aerated constantly, and water was changed once every 24 hours.

Except as otherwise noted, all egg masses used in a single experiment were carefully matched as to color to ensure that they were similar in age and developmental stage.

Fixing and Counting Procedure

At the end of the experiments, contents of the culture chambers were fixed in 10% neutral formalin/seawater (filtered) and stored in 15 ml glass vials (Fig. 13).

The large number of eggs in each sample necessitated subsampling prior to counting. The method used was modified from Frolander (1968). Vials of fixed eggs and larvae were rinsed into a graduated cylinder (50 or 100 ml depending upon how many organisms were in the sample) and diluted with 10% neutral formalin/seawater to a density of approximately 500 organisms/ml. The cylinder was stoppered, inverted three times, and a 1 ml sample removed immediately with a Stempel pipette. If any bubbles were found in the subsample after it had been drawn into the pipette it was replaced and the inversion and sampling procedure repeated.

The 1 ml subsample was washed into the trough of a plankton counting wheel and examined under a binocular dissecting microscope at 26-28X magnification. Numbers of unhatched eggs, stage I larvae and stage II larvae were counted (Fig. 14).

Figure 13. Fixed eggs and nauplii. Egg mass at left is still partially intact.

Figure 14. Counting apparatus. Center: Dissecting microscope with counting wheel on stage. Right foreground: Stempel pipette.

A minor problem occurred when, in some cases, clumps of the undissociated egg mass remained after fixing (Fig. 13). These would slowly fall apart after remaining in the formalin solution for a period of several weeks, but accurate counting of unhatched eggs was difficult until they had done so. Eventually I found that treatment of fixed egg mass clumps with an 0.25% trypsin solution after 3 min at 30° C would separate the eggs. The trypsin was inactivated after treatment by adding an equal volume of medium containing 10% fetal calf serum, and the separated eggs were washed several times with filtered seawater before being put back into the vial.

In experiments 5 and 6, subsamples were prepared and counted twice. Counts usually agreed well if the clumps were separated prior to counting.

Events Observed

Events observed included number of days from collection to hatching, hatching success, and stage I to stage II molting success. Hatching success was quantified with a hatching success index (Abbott 1975):

$$\frac{\text{no. stage I larvae + no. post-stage I larvae}}{\text{no. stage I larvae + no. post-stage I larvae + no. unhatched eggs}} \times 100$$

Molting success was quantified with a molting success index (Abbott and Mix 1979):

$$\frac{\text{no. post-stage I larvae}}{\text{no. stage I larvae + no. post-stage I larvae}} \times 100$$

The designation "post-stage I" was used because some post-stage I larvae were detected in a few instances. These were not counted as such because of difficulty in distinguishing them from stage II larvae and because their number would be severely affected by lack of food.

Water Analyses

Two series of analyses were conducted to determine background levels of phthalates in the synthetic seawater used in this study and to determine the extent to which dibutyl phthalate in the test solutions remained in solution between water changes.

Background tests were run on one liter samples of synthetic seawater which had been prepared and autoclaved in plastic (polypropylene) carboys, seawater prepared and autoclaved in glass bottles, and seawater prepared and autoclaved in glass after having been treated with activated charcoal.

Tests for phthalate loss between major changes were conducted by analyzing four days worth of effluent from culture chambers in experiment 6. Effluents of the same nominal DBP content were pooled, stored in tightly stoppered glass bottles under refrigeration, and extracted and analyzed immediately once the entire sample was collected.

Extraction Procedure

All samples were brought to room temperature prior to extraction. Samples were placed in a two-liter separatory funnel and shaken

vigorously for two minutes with two 200 ml portions of hexane. The hexane portions were pooled and concentrated to about 5 ml with a rotary evaporator, then rinsed into graduated centrifuge tubes with about 3 ml of additional hexane. The hexane extracts were evaporated under nitrogen to 1 or 0.5 ml, depending upon the expected phthalate concentration in the samples.

Chemical Analysis

Seawater extracts were analyzed using reverse-phase high performance liquid chromatography (HPLC) under the conditions shown in Table I. Calibration runs were made at 254 and 224 nm with a 50 ppm mixed standard of diethyl phthalate (DEP), dibutyl phthalate (DBP), diethylhexyl phthalate (DEHP) and dioctyl phthalate (DOP). Ratios of peak areas at these wavelengths were calculated and used to confirm the identity of peaks in the samples, which were also run at 254 and 224 nm.

Experimental Design

Six separate experiments were conducted in this study. Experiments 1-4 were designed to: identify optimal temperature and lighting regimes for the developing embryos; determine if they could be grown without antibiotics; and, identify a post-hatching end point when there would be sufficient numbers of post-stage I nauplii to draw valid conclusions regarding molting success without extending the experiment for so long that a significant number of early molting stage II nauplii died from starvation.

TABLE I. HPLC OPERATING CONDITIONS USED TO ANALYZE WATER SAMPLES FOR PHTHALATES.

Liquid Chromatograph	Spectraphysics 8000, Valco injection valve with 100 µl loop
Columns	Guard: Alltech 10 cm x .32 cm Vydoc 201 TP 10µ C_{18} packing
	Analytical: Perkin-Elmer HC-ODS PAH, 25 x .26 cm 10µ C_{18} packing
Mobile Phase	Acetonitrile in water: 45-100%, 2.75%/min. 100%, 10 min. 100-45% equilibration, 20 min.
	1 ml/min. flow rate temperature 30° C
Detector	Schoeffel 1770 variable wavelength UV detector 254 nm, range .02 224 nm, range .2

The first investigator to successfully raise Pollicipes larvae in vitro had best results from incubating egg mass pieces in total darkness at natural (14° C) temperatures (Lewis 1975a). In a later study (Mix et al. 1979) it was suggested that maintaining eggs and larvae under ambient laboratory temperatures and lighting conditions would considerably simplify the culture procedure. Their results demonstrated that embryos developed normally when maintained at room temperature (21-25° C air, 16-19° C inside culture chamber) and that mean hatching times did not differ significantly between embryos raised in total darkness and those raised under ambient laboratory lighting cycles.

Experiments 1 and 2 were essentially repetitions using the conditions described above. Bright orange egg masses were used since they had been used in that study. Egg mass quarters were grown under four different temperature and lighting regimes (Table II). Terms used to describe these conditions are described as follows: ambient temperature was ambient laboratory temperature, which ranged from 20-31° C while this study was in progress; cold was 14° ± 1° C (Fig. 15); light was ambient laboratory lighting (approximately 10 hours light, 14 hours dark over 24 hours); and dark was total darkness. Ten mg/l of penicillin and streptomycin were added to the seawater used in these experiments. In experiment 1, the culture chamber contents were fixed three days after hatching was first observed in the chamber. In experiment 2, the contents were fixed when the egg mass began to visibly disintegrate.

TABLE II. EXPERIMENTAL PROTOCOL, EXPERIMENTS 1-6.

Exp. 1 - 1A Ambient temp./light
1B Cold/light
1C Cold/dark
1D Ambient temp./dark

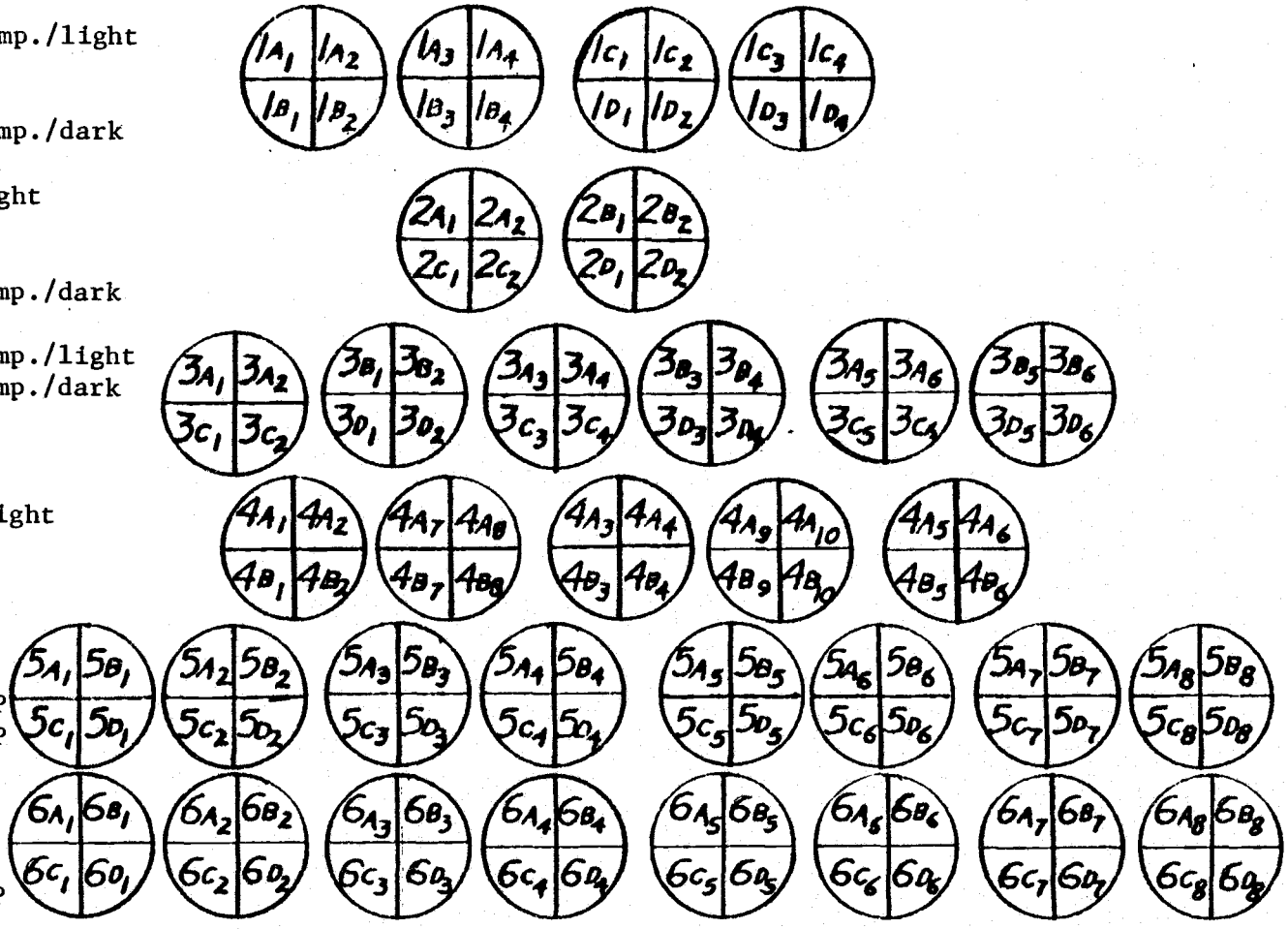
Exp. 2 - 2A Ambient/light
2B Cold/light
2C Cold/dark
2D Ambient temp./dark

Exp. 3 - 3A Ambient temp./light
3B Ambient temp./dark
3C Cold/light
3D Cold/dark

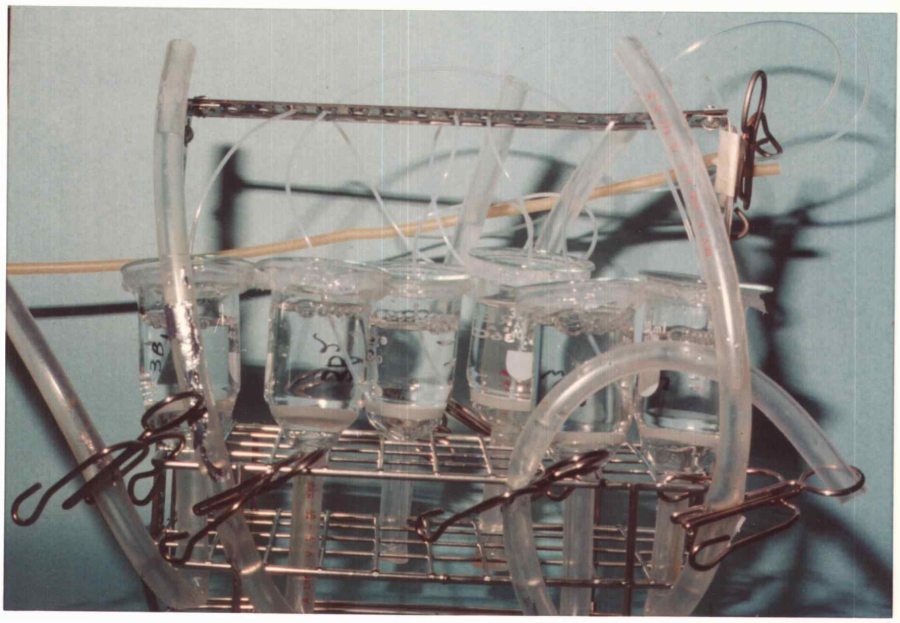
Exp. 4 - 4A Cold/low light
4B Cold/light

Exp. 5 - 5A 0 PPB DBP
5B 10 PPB DBP
5C 100 PPB DBP
5D 1000 PPB DBP

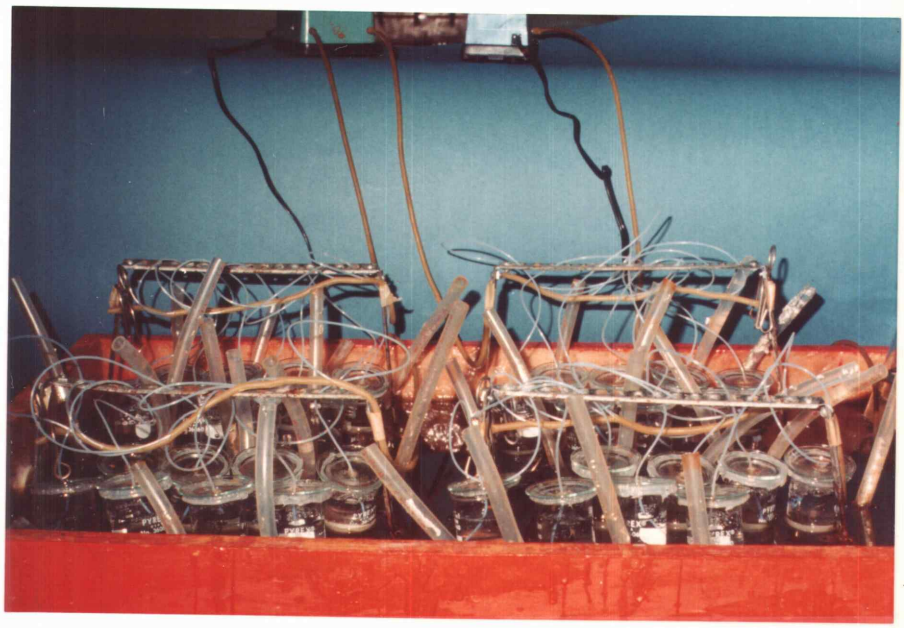
Exp. 6 - 6A 0 PPB DBP
6B 10 PPB DBP
6C 100 PPB DBP
6D 1000 PPB DBP



5 and 6 all grown under cold/low light conditions.



a



b

Figure 15. Culture chambers in use. a. At ambient temperature. b. At 14° C.

Experiment 3 (Table II) was similar to 1 and 2 with the following exceptions: antibiotics were not used in this experiment nor in any later ones, and light orange egg masses from a total of three adults were used (Fig. 16). The three-day end point was used in this and in all subsequent experiments.

Experiment 4 (Table II) involved only cold conditions, and egg mass pairs of three different stages (white-orange, light orange and bright orange) were used. Dark conditions were replaced by "low light": racks of culture chambers were covered with a double layer of thin black plastic which admitted a very small amount of light when laboratory lights were on. This was done as an attempt to more closely simulate lighting conditions inside the adults' mantle cavity. When the tide is in and the barnacles are feeding, the mantle cavity is probably not in total darkness as it is when the tide is out and the barnacles are tightly closed.

Experiments 5 and 6 (Table II) involved exposing egg mass quarters from four adults to concentrations of 0, 100, and 1000 ppb DBP. Cold, low-light conditions and the three-day post-hatching end point were used in both experiments.

In experiment 5, a 100-ppm stock solution of DBP was prepared by dissolving 0.1 g DBP in 15 ml re-distilled 95% ethanol and adding it to 985 ml of prepared seawater. Test solutions were made up one liter at a time by adding appropriate amounts of this stock solution to prepared seawater. Although it was desirable to use light orange egg masses because of the longer period of development during which

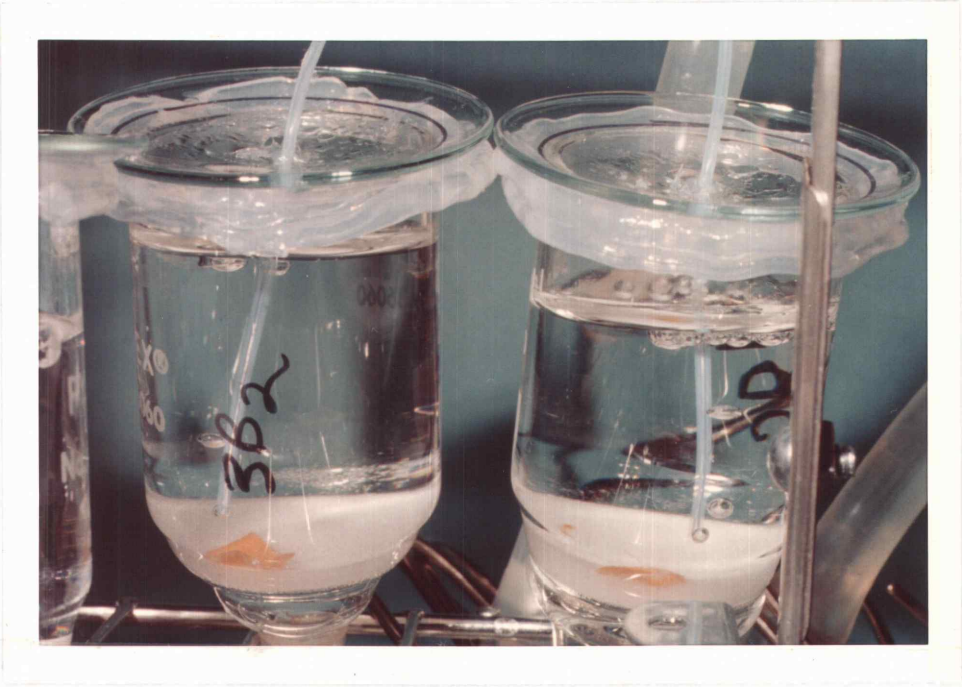


Figure 16. Close-up of culture chamber containing egg mass quarter at light orange stage.

they could be exposed to DBP, it was late in the brooding season by the time this experiment was performed and only bright orange eggs were available.

Experiment 6 was a refined version of experiment 5. Light orange egg masses were obtained, and the stock solution used to make the test solutions was 1000 ppm DBP dissolved in spectrograde acetone. Only 500 ml of the DBP test solution was made up at a time, since this amount lasted about two days and it was reported (Laughlin et al. 1978) that DBP in the range of concentrations used here remained in solution in seawater for at least 48 hours if it was carefully stored.

In an attempt to reduce variation between culture chambers, the chambers were examined for evidence of hatching every eight hours instead of every 24 as in the previous experiments. The criteria for hatching used here was when at least five nauplii were observed swimming in the chamber. The three-day end point was measured as 72 hours from the time of hatching.

Table II summarizes the experimental protocols for the six experiments (p. 31).

Statistical Analyses

In most of the experiments (1-5), variances were not homogeneous between treatment groups as indicated by an F-Max test (Sokal and Rohlf 1969); therefore, a randomized block design two-way analysis of variance was used to analyze the data (Sokal and Rohlf 1969).

Analyses of variance (ANOVAs) for hatching and molting indices were performed on an Hewlett-Packard 85 computer with egg mass quarters designated as "rows" and treatment groups as "columns."

In experiment 6, the variances were homogeneous according to the F-Max test, so a regular two-way ANOVA was also performed to determine if there were significant differences in hatching or molting success between different adult barnacles. In these tests, the two egg-mass quarters in each treatment group that came from a single adult were considered as replications of a single row unit.

When F values indicated significant differences between groups being examined, the Student-Newman-Keuls test (Sokal and Rohlf 1969) was employed to identify which group(s) were different.

Arcsine transformations were used on the data because both hatching and molting indices are expressed as proportions (Sokal and Rohlf 1969).

RESULTS

Experiment 1

Eggs grown at 14° C took an average of eight days longer to hatch than those grown at ambient temperatures (Fig. 17). Very little difference in mean time to first hatch (about four days) was found between eggs grown at similar temperatures under light versus dark conditions. Neither hatching nor molting successes were significantly different under any of the temperature and lighting combinations. For hatching, observed $F = 0.0 < F_{(.05,3,9)} = 3.86$. For molting, observed $F = 0.6 < F_{(.05,3,9)} = 3.86$. Hatching and molting data are summarized in Table III.

Experiment 2

Virtually no difference in time to first hatch was found between any of the groups (Fig. 17). That may have been because fewer egg masses (16 quarters from two adults) were used, resulting in less overall variance. Also, these eggs were closer to hatching when collected than those used in experiment 1 and spent less time in the different temperature regimes.

The C group (cold/dark) had a significantly lower hatching success than the others: observed $F = 14.6 > F_{(.05,3,3)} = 9.28$. This group also had a numerically lower mean molting success but the difference was not significant: observed $F = 0.4 < F_{(.05,3,3)} = 9.28$. Hatching and molting data from experiment 2 are summarized in Table IV.

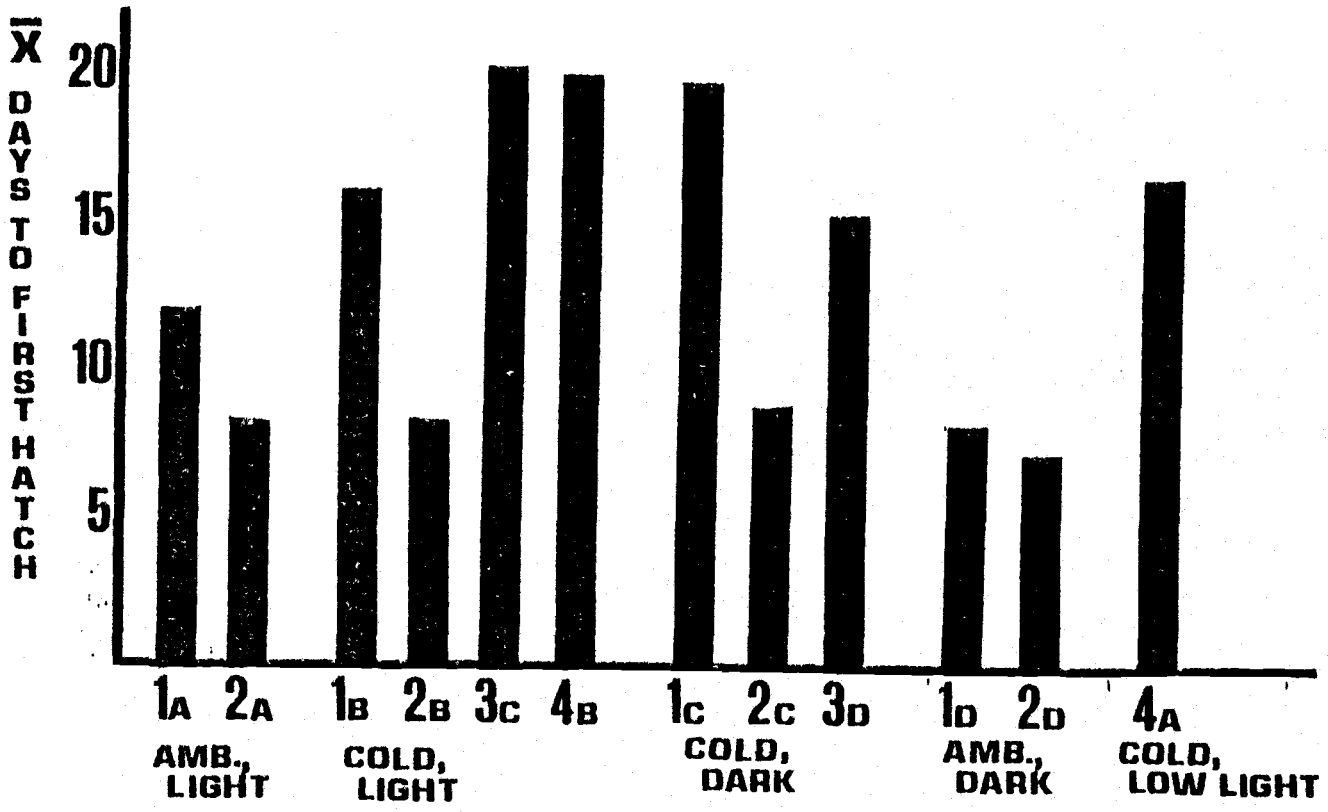


Figure 17. Mean number of days from collection to first hatch, experiments 1-4.

TABLE III. HATCHING AND MOLTING DATA, EXPERIMENT 1.

Expt. no.	Hatching index		Molting index		Mean, hatching index		Mean, molting index	
	percent	transformed (asn $\sqrt{n}$)	percent	transformed (asn $\sqrt{n}$)	percent	transformed	percent	transformed
1A1	82.96	65.62	15.18	22.93	$\bar{X}$ = 76.53	$\bar{X}$ = 62.13	$\bar{X}$ = 11.82	$\bar{X}$ = 19.81
1A2	82.88	65.56	9.24	17.70	S^2 = 269.0		S^2 = 17.17	
1A3	48.76	44.29	6.41	14.67				
1A4	91.51	73.06	16.46	23.94				
1B1	78.19	62.16	9.42	17.87	$\bar{X}$ = 78.29	$\bar{X}$ = 62.95	$\bar{X}$ = 8.45	$\bar{X}$ = 15.86
1B2	61.51	51.65	4.27	11.93	S^2 = 118.39		S^2 = 36.27	
1B3	81.74	64.70	17.85	24.99				
1B4	91.71	73.27	2.26	8.65				
1C1	78.83	62.21	11.24	19.59	$\bar{X}$ = 75.25	$\bar{X}$ = 61.79	$\bar{X}$ = 10.17	$\bar{X}$ = 17.16
1C2	40.00	39.23	22.92	28.60	S^2 = 439.82		S^2 = 65.50	
1C3	92.11	73.69	2.08	8.29				
1C4	90.07	71.63	4.44	12.16				
1D1	75.78	60.52	6.88	15.21	$\bar{X}$ = 75.08	$\bar{X}$ = 60.48	$\bar{X}$ = 6.76	$\bar{X}$ = 13.44
1D2	88.64	70.30	7.70	16.11	S^2 = 77.90		S^2 = 0.4	
1D3	71.54	57.76	6.49	14.76				
1D4	64.37	53.35	5.97	14.14				

TABLE IV. HATCHING AND MOLTING DATA, EXPERIMENT 2.

Expt. no.	Hatching index		Molting index		Mean, hatching index		Mean, molting index	
	percent	transformed (asn $\sqrt{n}$)	percent	transformed (asn $\sqrt{n}$)	percent	transformed	percent	transformed
2A1	96.40	68.36	26.05	30.69	$\bar{X}$ = 90.37	$\bar{X}$ = 72.29	$\bar{X}$ = 18.60	$\bar{X}$ = 25.10
2A2	94.33	76.22	11.15	19.51	S^2 = 15.60		S^2 = 55.50	
2B1	78.77	62.56	2.67	9.40	$\bar{X}$ = 75.80	$\bar{X}$ = 50.58	$\bar{X}$ = 24.14	$\bar{X}$ = 25.94
2B2	72.82	58.58	45.61	42.48	S^2 = 8.90		S^2 = 265.90	
2C1	53.32	46.90	6.38	14.63	$\bar{X}$ = 57.47	$\bar{X}$ = 49.17	$\bar{X}$ = 4.19	$\bar{X}$ = 11.38
2C2	61.12	51.43	2.00	8.13	S^2 = 17.23		S^2 = 244.45	
2D1	85.21	67.38	7.97	16.40	$\bar{X}$ = 89.58	$\bar{X}$ = 71.57	$\bar{X}$ = 15.02	$\bar{X}$ = 22.22
2D2	93.95	75.76	22.08	28.03	S^2 = 19.10		S^2 = 195.86	

It was decided during this experiment that an end point based on disintegration of the egg mass was not practical. Egg mass disintegration did not seem to follow the same pattern in different egg masses; in some, the egg mass would fall apart within a day or two of when the first eggs hatched, while in others, lumps of the intact egg mass remained four days later.

Experiment 3

This experiment empirically demonstrated the need for a cooling system, at least in this laboratory (Weniger Hall 422). Hot summer weather caused the room temperature to rise above 30° C and none of the ambient temperature groups (3A, 3B) showed any signs of hatching.

The 14° C groups (3C, 3D) had no significant differences in hatching or molting success between light and dark conditions. For hatching, observed $F = 2.5 < F_{(.05,1,4)} = 7.71$. For molting, observed $F = 4.0 < F_{(.05,1,4)} = 7.71$. Hatching and molting data are summarized in Table V. Mean hatching times were not appreciably different (Fig. 17).

Experiment 4

In this experiment, an attempt was made to more closely simulate lighting conditions within the adult barnacle by alternating darkness with periods of very low light. Another group was grown under ambient lighting conditions and both were maintained at 14° C. Each group contained egg mass quarters with different color stages. Both

TABLE V. HATCHING AND MOLTING DATA, EXPERIMENT 3.

Expt. no.	Hatching index		Molting index		Mean, hatching index		Mean, molting index	
	percent	transformed (asn $\sqrt{n}$)	percent	transformed (asn $\sqrt{n}$)	percent	transformed	percent	transformed
3C1	78.29	62.23	48.91	44.38	$\bar{X}$ = 82.06	$\bar{X}$ = 64.98	$\bar{X}$ = 41.98	$\bar{X}$ = 39.75
3C2	83.61	66.12	61.15	51.54	S^2 = 5.40		S^2 = 301.29	
3C3	84.41	66.74	36.04	36.89				
3C4*	---	---	---	---				
3C5	80.44	63.75	11.25	19.60				
3C6	83.55	66.07	52.53	46.45				
3D1	64.73	53.57	41.80	40.28				
3D2	79.86	63.33	17.30	24.58	$\bar{X}$ = 68.25	$\bar{X}$ = 57.73	$\bar{X}$ = 20.16	$\bar{X}$ = 27.11
3D3	45.58	42.46	2.02	8.17	S^2 = 198.76		S^2 = 273.20	
3D4	56.49	48.73	3.42	10.66				
3D5	76.61	61.08	13.66	21.69				
3D6	86.23	68.22	42.77	40.84				

*3C4 was lost when drainage tube came loose from chamber.

light orange and bright orange egg masses had been grown successfully in previous experiments but culturing of white-orange eggs had not yet been attempted and it was desirable to see how they compared with the others.

Very little difference was seen in number of days to first hatch between the two lighting conditions (Fig. 17). Although the cold/low light group had hatching and molting successes similar to eggs in previous experiments (Figs. 18, 19), the cold/light group had such a low hatching success that three days after hatching began, it was difficult to find any nauplii in an adequate subsample, so this group was not counted. This hatching failure was perplexing, since eggs had been raised under similar conditions in all of the previous experiments without showing negative effects. Apparently the first few eggs began to hatch well in advance of the rest. In subsequent experiments a stricter criterion was used to determine the start of hatching: at least five active nauplii had to be observed in the chamber.

No differences were found in hatching or molting success between white-orange egg masses and the others (Fig. 20). For hatching differences between white-orange and light orange eggs, observed $F = 0.1 < F_{(.05,1,3)} = 10.1$. For molting, observed $F = 0.0 < F_{(.05,1,3)} = 10.1$. Hatching and molting data are summarized in Table VI.

Patterns of hatching and molting success from all of the preliminary experiments and the controls in experiment 5 are compared

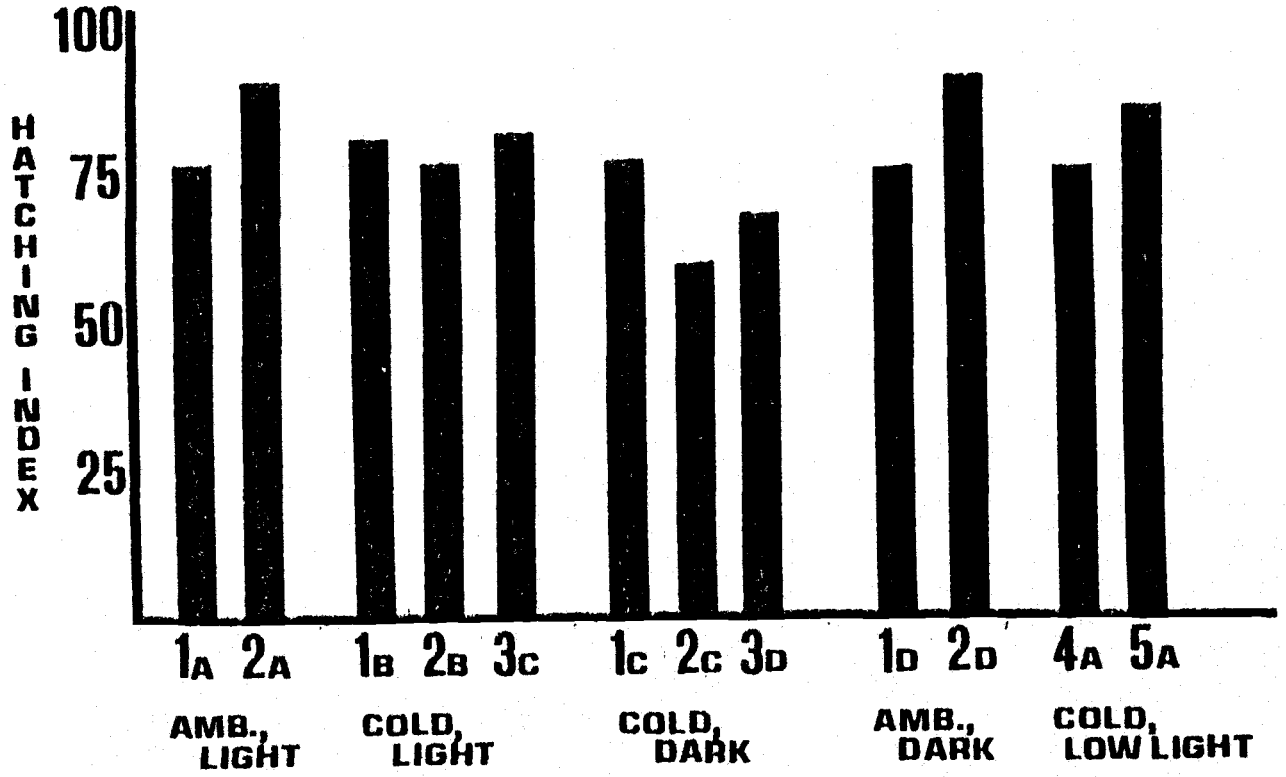


Figure 18. Comparison of hatching indices, experiments 1-4 and 5A.

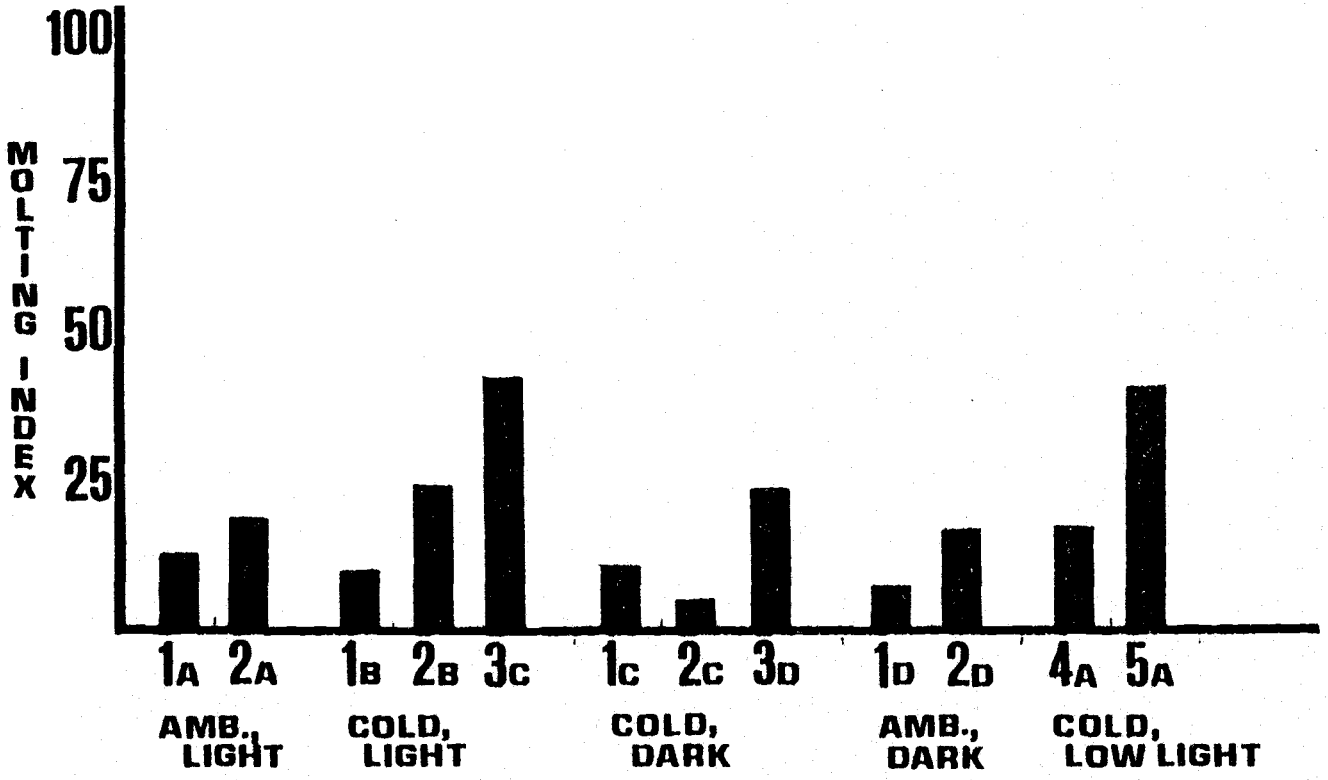


Figure 19. Comparison of molting indices, experiments 1-4 and 5A.

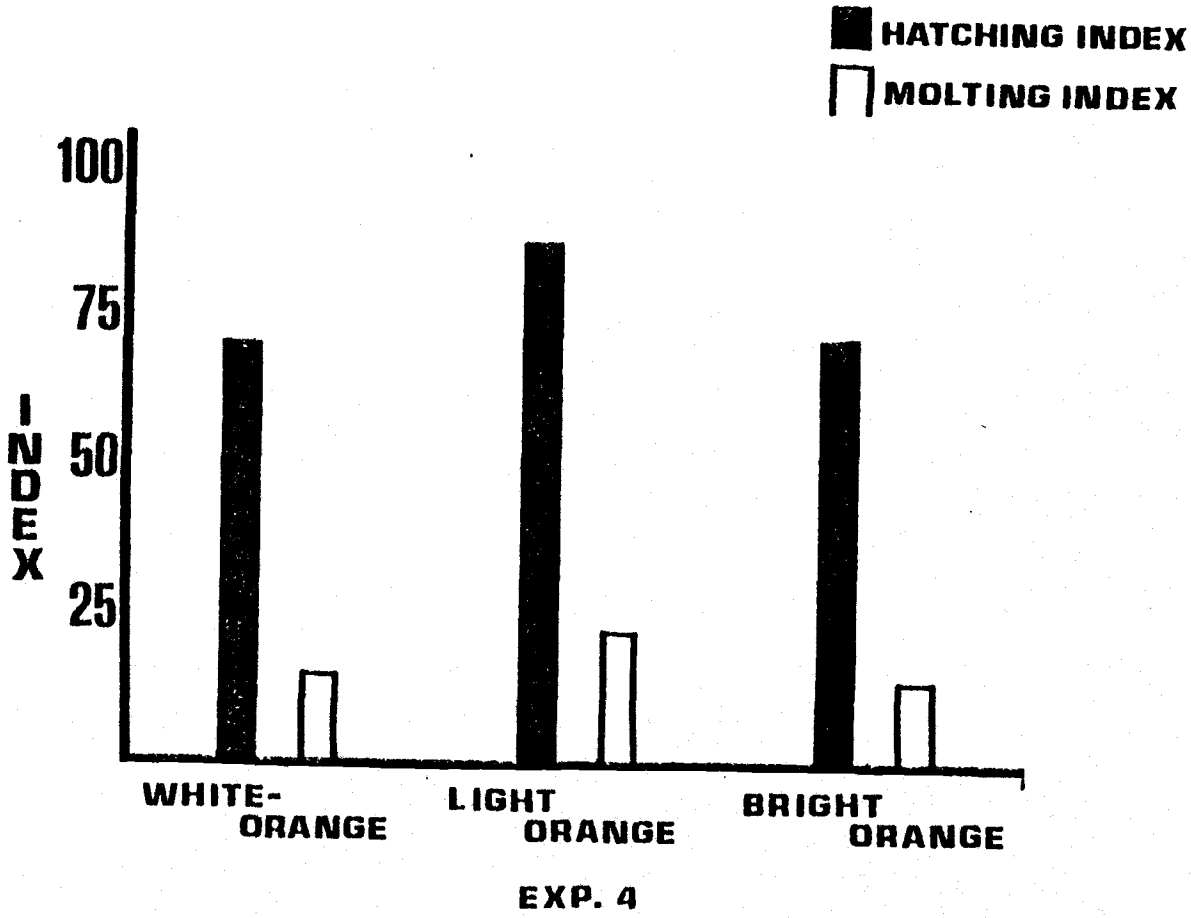


Figure 20. Hatching and molting indices from experiment 4 broken down by stage of egg mass when collected.

TABLE VI. HATCHING AND MOLTING DATA, EXPERIMENT 4.

Expt. no.	Hatching index		Molting index	
	percent	transformed (asn $\sqrt{n}$)	percent	transformed (asn $\sqrt{n}$)
4A1	79.09	62.79	4.46	12.19
4A2	86.08	68.09	1.69	7.47
4A3	90.09	71.65	25.08	30.05
4A4	91.39	72.94	34.55	36.00
4A5	42.77	40.84	5.92	14.08
4A6	84.81	67.06	19.40	26.13
4A7	74.64	59.76	49.51	44.72
4A8	38.98	38.63	1.24	6.39
4A9	81.39	64.44	19.38	26.12
4A10	82.34	65.15	5.50	13.56

Hatching		Molting	
percent	transformed	percent	transformed
$\bar{X} = 75.16$	$\bar{X} = 61.09$	$\bar{X} = 13.59$	$\bar{X} = 21.67$
$S^2 = 233.56$	$S^2 = 128.20$	$S^2 = 158.82$	$S^2 = 149.49$

in Figures 18 and 19. No trends in hatching or molting success related to temperature or lighting conditions can be observed, discounting the instance when the ambient temperature exceeded the tolerance range of the eggs.

I concluded that any benefit from the reduced development time under ambient laboratory temperatures was not worth the risk of losing all the eggs should the temperature suddenly rise; therefore all subsequent experiments were performed at 14° C. The low-light conditions from experiment 4 were used in order to simulate the natural surroundings of the eggs.

Experiment 5

In this experiment egg mass quarters were exposed to concentrations of 0, 10, 100 and 1000 ppb dibutyl phthalate.

No difference in time to first hatch was observed between the control group and any of the treatment groups (Fig. 21).

Hatching successes in DBP exposure groups were not significantly different from the controls. However, a significant decrease in molting success was observed in the group exposed to 1000 ppb DBP (Fig. 22). For hatching, observed $F = 2.5 < F_{(.05,3,21)} = 3.07$. For molting, observed $F = 4.8 > F_{(.05,3,21)} = 3.07$. Hatching and molting data are summarized in Table VII.

The within-treatment variance in molting success increased markedly at the 10 and 100 ppb exposure levels; variances in hatching success increased to a lesser degree (Table VIII). Although variances in previous experiments tended to be large and heterogeneous

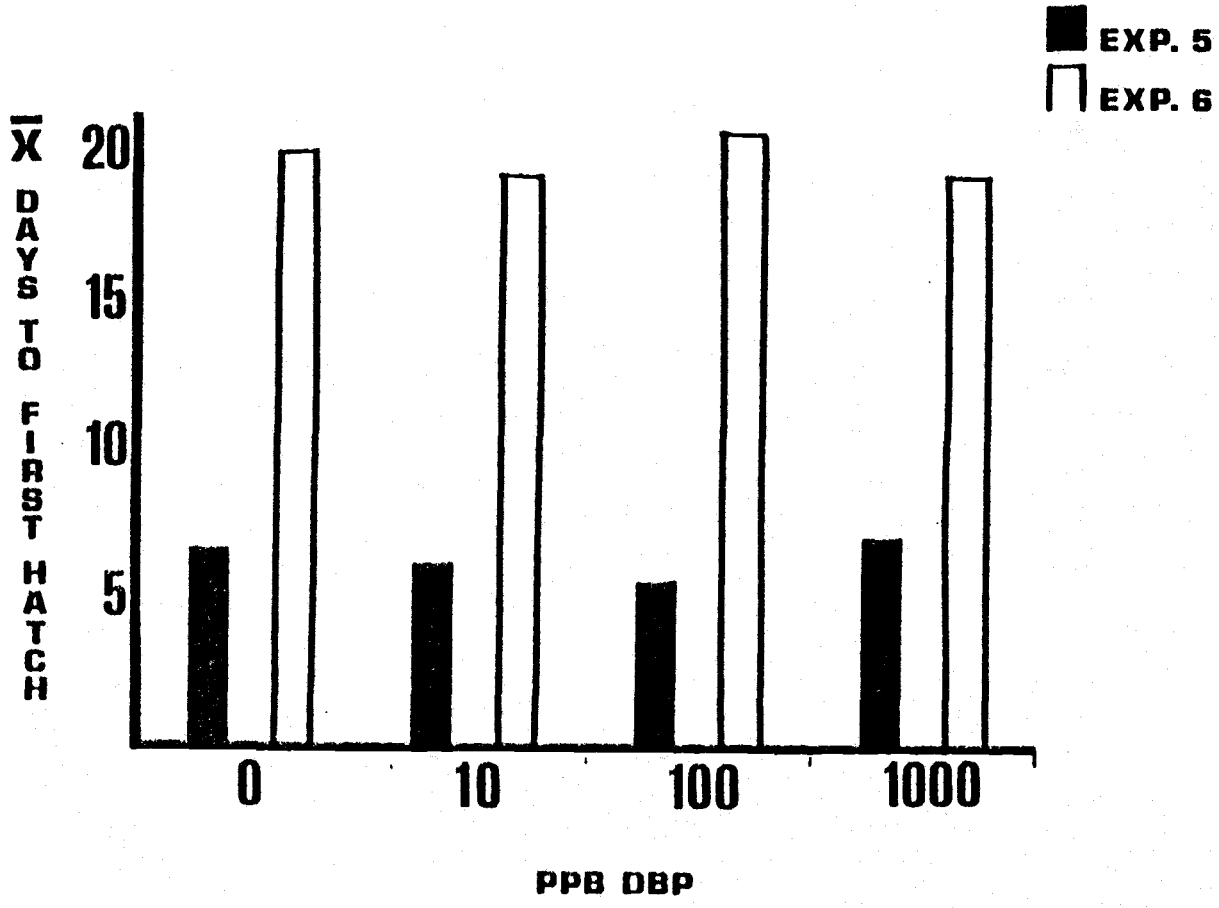


Figure 21. Mean number of days from collection to first hatch, experiments 5-6.

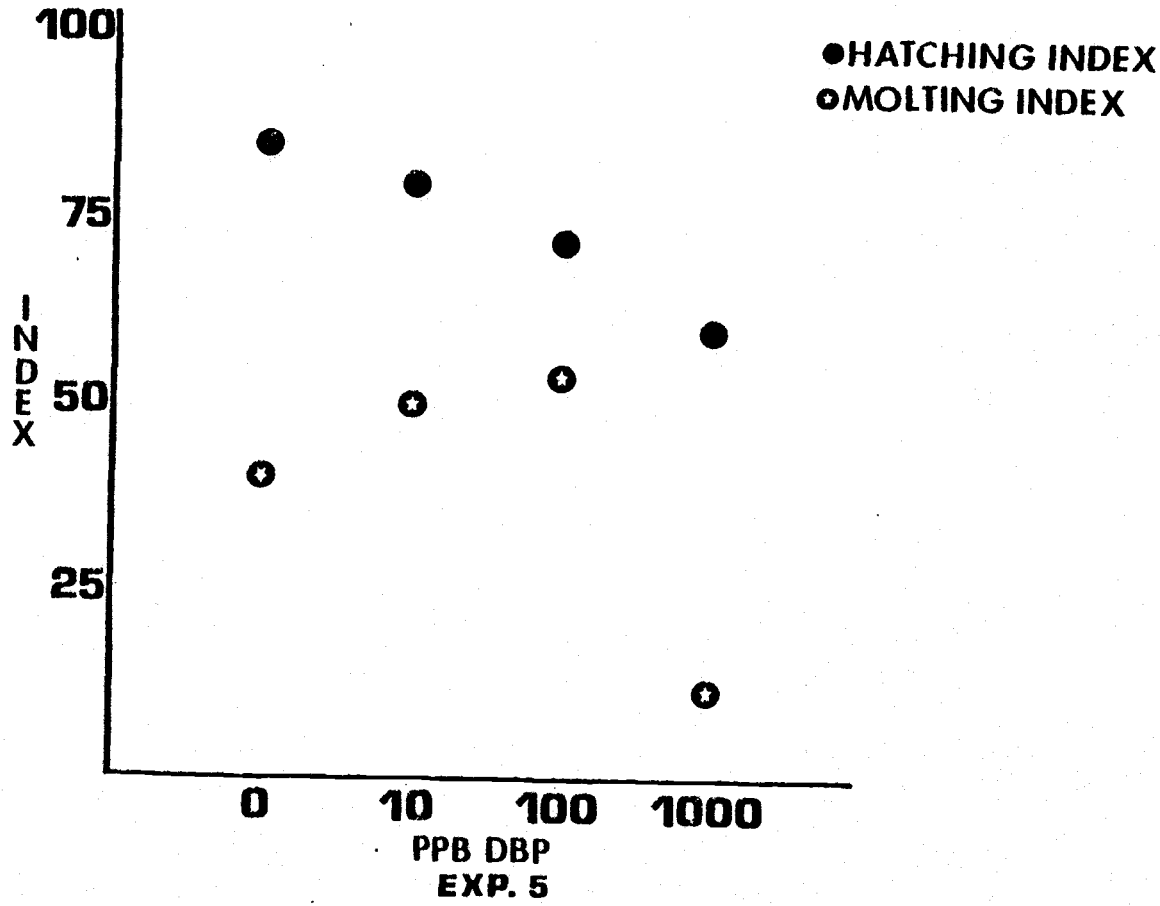


Figure 22. Hatching and molting indices at different DBP exposures, experiment 5.

TABLE VII. HATCHING AND MOLTING DATA, EXPERIMENT 5.

Expt. no.	Hatching index %	Hatching index asn $\sqrt{n}$	Molting index %	Molting index asn $\sqrt{n}$
5A1	75.65	60.43	46.96	43.26
5A2	80.37	63.70	33.66	35.46
5A3	88.32	70.02	24.63	29.75
5A4	90.94	72.48	62.60	52.30
5A5	80.14	63.54	55.99	48.44
5A6	93.54	75.28	59.55	50.51
5A7	82.87	65.55	22.08	28.03
5A8	80.19	63.57	21.21	27.42
5B1	65.19	53.84	10.22	18.64
5B2	46.84	43.19	4.63	12.43
5B3	95.80	78.17	81.17	64.28
5B4	95.07	77.17	79.77	63.27
5B5	92.58	74.19	77.24	61.51
5B6	82.29	65.11	39.67	39.04
5B7	51.49	45.85	17.95	25.07
5B8	98.97	84.18	84.26	66.63

	Hatching	Molting
	%	%
	$\overline{X}$ = 84.00	$\overline{X}$ = 40.84
	S^2 = 33.93	S^2 = 267.77
Asn	$\overline{X}$ = 66.82	$\overline{X}$ = 39.40
	%	%
	$\overline{X}$ = 78.62	$\overline{X}$ = 49.36
	S^2 = 387.04	S^2 = 1068.14
Asn	$\overline{X}$ = 65.21	$\overline{X}$ = 43.86

TABLE VII. HATCHING AND MOLTING DATA, EXPERIMENT 5 (Continued).

Expt. no.	Hatching index %	Hatching index asn $\sqrt{n}$	Molting index %	Molting index asn $\sqrt{n}$
5C1	50.43	45.25	14.88	22.69
5C2	78.95	62.29	66.65	54.73
5C3	97.10	80.20	84.28	66.64
5C4	97.13	80.25	80.73	63.96
5C5	98.13	82.14	88.66	70.32
5C6	96.37	79.02	87.28	69.11
5C7	39.06	38.68	5.43	13.48
5C8	32.66	34.85	4.03	11.58
5D1	67.30	55.12	12.88	21.03
5D2	38.49	38.35	6.25	14.48
5D3	52.30	46.32	4.21	11.84
5D4	32.15	34.54	26.21	30.79
5D5	70.48	57.09	5.34	13.36
5D6	80.53	63.82	20.73	27.08
5D7	59.06	50.22	4.85	12.72
5D8	76.26	60.84	12.23	20.47

	Hatching	Molting
	%	%
	$\bar{X}$ = 73.73	$\bar{X}$ = 53.99
	S^2 = 707.57	S^2 = 1310.84
Asn		
	$\bar{X}$ = 62.89	$\bar{X}$ = 46.56
	%	%
	$\bar{X}$ = 59.57	$\bar{X}$ = 11.59
	S^2 = 268.25	S^2 = 58.35
Asn		
	$\bar{X}$ = 50.79	$\bar{X}$ = 18.97

TABLE VIII. WITHIN-TREATMENT VARIANCES OBSERVED IN EXPERIMENTS 5 AND 6.

	Experiment 5		Experiment 6	
ppb DBP	S^2 hatching index	S^2 molting index	S^2 hatching index	S^2 molting index
0	33.93	267.77	277.73	14.56
10	387.04	1068.14	515.84	29.40
100	707.57	1310.84	500.82	9.72
1000	268.25	58.35	653.77	17.80

(Tables III-VI), both magnitude and heterogeneity of the molting success variances in experiment 5 were considerably greater.

Experiment 6

To determine if the increased variances noticed in experiment 5 were caused by exposure to the phthalate, experiment 6 was designed to reduce the occasional large "background" variance found in the preliminary experiments. The eggs were examined for hatching every eight hours instead of every 24, and fixed 72 hours after hatching had begun.

Variances in molting success were much smaller in experiment 6 than they had been in 5, and both hatching and molting variances were homogeneous, allowing a regular two-way analysis of variance to be performed to determine if there were significant differences in hatching or molting success between adults. A highly significant difference in molting success (observed $F = 21.1 > F_{(.05,3,16)} = 3.24$) and a significant difference in hatching success (observed $F = 4.6 > F_{(.05,3,16)} = 3.24$) between adults was found.

Unfortunately, 72 hours, when measured more accurately from time of first hatching as in this experiment, was not enough time for a reasonable number of the controls to molt. As a result, the overall molting success was so low that any DBP effects, if present, were not measurable (Fig. 23). No significant differences were found with hatching success: observed $F = 2.2 < F_{(.05,3,21)} = 3.07$. Hatching and molting data are summarized in Table IX.

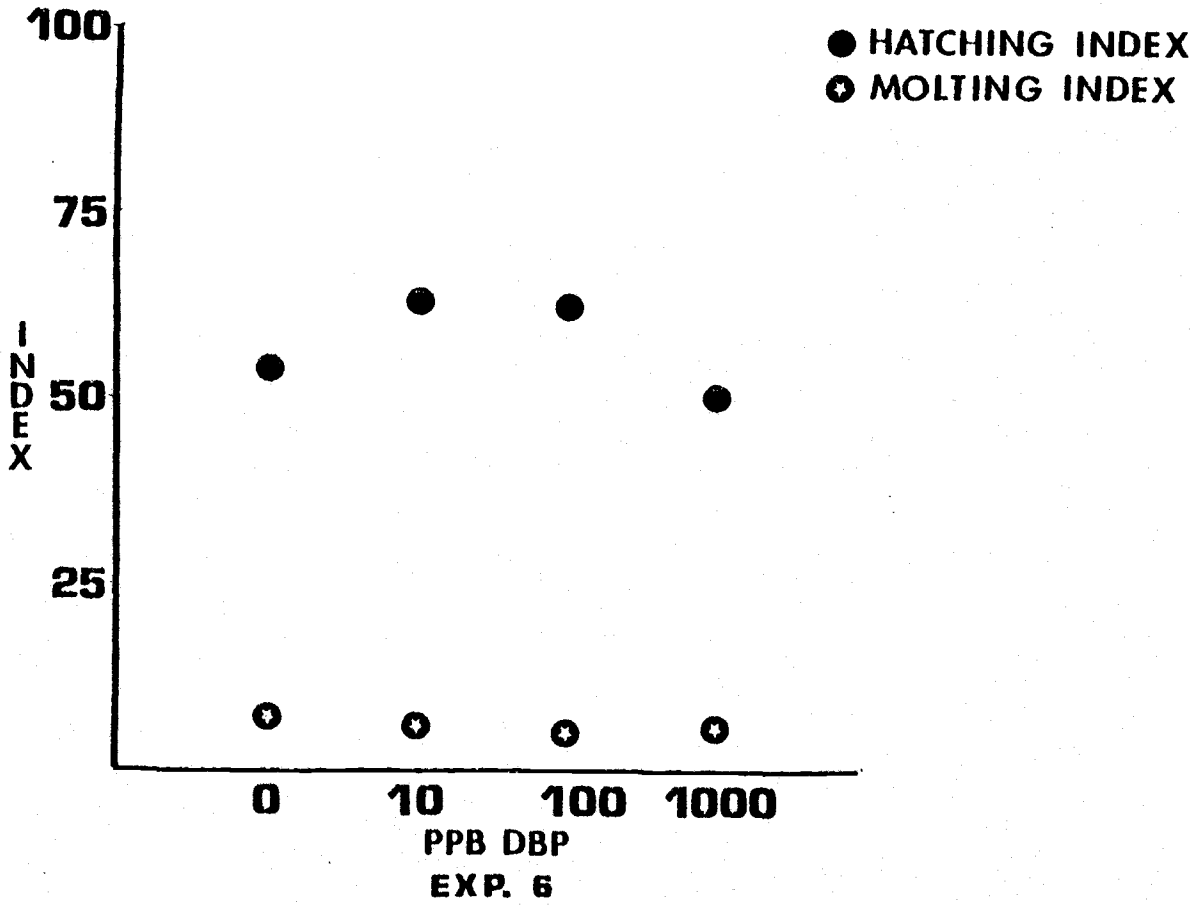


Figure 23. Hatching and molting indices at different DBP exposures, experiment 6.

TABLE IX. HATCHING AND MOLTING DATA, EXPERIMENT 6.

Expt. no.	Hatching index %	Hatching index asn $\sqrt{n}$	Molting index %	Molting index asn $\sqrt{n}$
6A1	53.10	56.78	4.05	11.61
6A2	66.47	54.62	2.67	9.40
6A3	34.22	35.80	3.40	10.63
6A4	39.08	38.69	5.59	13.68
6A5	71.38	57.66	5.55	13.63
6A6	77.19	61.47	8.35	16.80
6A7	29.53	32.92	7.28	15.65
6A9	57.79	49.48	15.47	23.16
6B1	80.47	63.77	19.94	26.52
6B2	69.47	56.46	6.01	14.19
6B3	40.35	39.44	2.39	8.89
6B4	35.15	36.36	4.17	11.78
6B5	97.41	80.74	7.87	16.29
6B6	89.24	70.85	5.54	13.61
6B7	40.02	39.24	2.83	9.68
6B8	53.48	47.00	2.52	9.13

	Hatching	Molting
	%	%
	$\bar{X}$ = 53.60	$\bar{X}$ = 6.55
	S^2 = 277.73	S^2 = 14.56
Asn	$\bar{X}$ = 47.18	$\bar{X}$ = 14.32
	%	%
	$\bar{X}$ = 63.20	$\bar{X}$ = 6.41
	S^2 = 515.84	S^2 = 29.40
Asn	$\bar{X}$ = 54.23	$\bar{X}$ = 13.76

TABLE IX. HATCHING AND MOLTING DATA, EXPERIMENT 6 (Continued).

Expt. no.	Hatching index %	Hatching index asn $\sqrt{n}$	Molting index %	Molting index asn $\sqrt{n}$
6C1	68.72	55.99	4.96	12.87
6C2	69.90	56.73	4.58	12.36
6C3	54.71	47.70	0.61	4.48
6C4	44.53	41.86	1.55	7.15
6C5	86.44	68.39	5.79	13.92
6C6	94.37	76.27	5.53	13.60
6C7	18.45	25.44	10.31	18.73
6C8	63.07	52.58	9.26	17.72
6D1	54.39	47.52	14.11	22.06
6D2	75.24	60.16	7.87	16.29
6D3	18.08	25.16	1.34	6.65
6D4	42.75	40.83	0.38	3.53
6D5	60.68	51.17	6.41	14.67
6D6	93.55	75.29	1.40	6.80
6D7	42.93	40.94	6.61	14.90
6D8	12.03	20.29	6.02	14.20

	Hatching	Molting
	%	%
	$\bar{X}$ = 62.52	$\bar{X}$ = 5.32
	S^2 = 500.82	S^2 = 9.72
Asn	$\bar{X}$ = 53.12	$\bar{X}$ = 12.60
	%	%
	$\bar{X}$ = 49.96	$\bar{X}$ = 5.52
	S^2 = 653.77	S^2 = 17.80
Asn	$\bar{X}$ = 45.17	$\bar{X}$ = 12.39

Phthalate Background Tests

Synthetic seawater prepared in plastic and glass contained unacceptably high levels of DBP: 8.8 and 20.9 ppb, respectively (compare Fig. 24 with Fig. 25). DEHP, DEP, and DOP were also present, with DEHP found in larger amounts (6.7-11.2 ppb) than the other two (Fig. 25a,b).

Filtering glass-prepared water through activated charcoal removed nearly all the DBP but the DEHP content of this water was even higher than the others (25.8 ppb) (Fig. 25c).

I decided to charcoal-filter water used in subsequent experiments despite the presence of DEHP since DBP is the more toxic of the two and DEHP was found not to affect development of larval marine crustaceans at concentrations of up to 1000 ppb (Laughlin et al. 1978).

DBP Remaining Between Water Changes

Analysis of culture chamber effluents in experiment 6 revealed that the loss of DBP from solution increased with the initial DBP concentration (Table X). Seawater extracts from the 10, 100, and 1000 ppb effluents contained 9.4, 52.6, and 157.3 ppb DBP. The control extract (0 ppb added) contained 1.8 ppb DBP, indicating little if any contamination had occurred after the water was filtered.

DEHP remained a persistent contaminant in the samples. Levels ranged from 9.9 to 25.3 ppb. It is interesting to note that DEHP

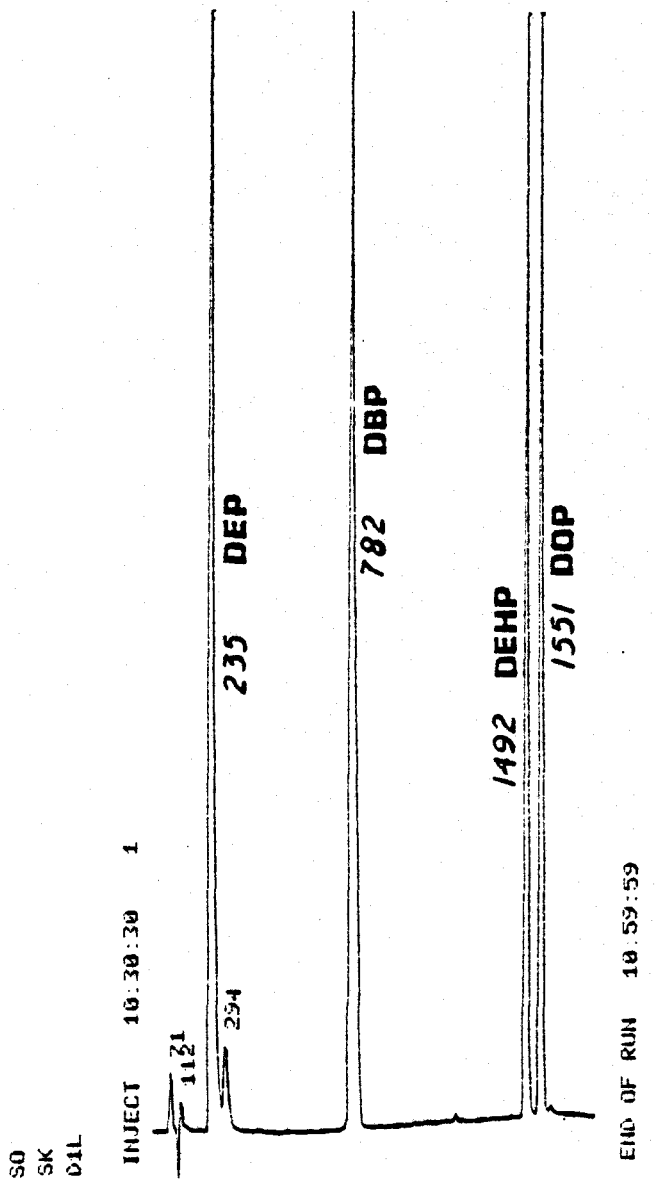


Figure 24. HPLC calibration run, 50 ppm mixed standard at 224 nm. Vertical numbers indicate retention times.

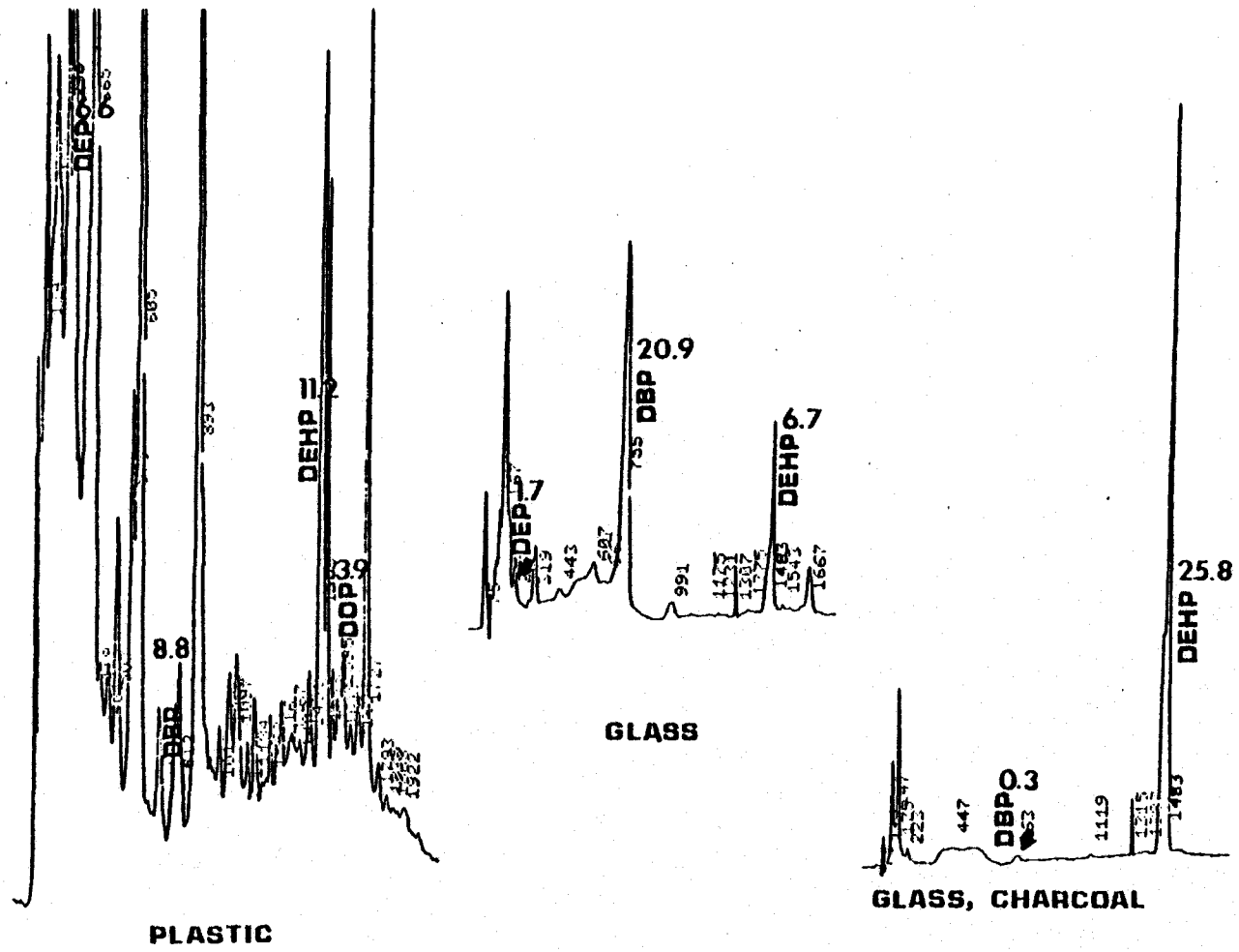


Figure 25. HPLC runs for phthalate background tests. Horizontal numbers indicate phthalate content in ppb, vertical numbers indicate retention times.

TABLE X. DBP LOSS AFTER 24 HOURS IN CULTURE CHAMBER.

ppb DBP added to water	ppb DBP after 24 hours	Change in concentration ppb	Change in concentration %
0	1.8	+ 1.8	-
10	9.4	- 0.6	- 6.0
100	52.6	- 47.4	- 47.4
1000	157.3	- 842.7	- 84.3

levels seemed to be highest when DBP contamination was low (Fig. 26); this was also noticed in the background test samples (Fig. 25).

DEP content remained constant around 4 ppb and DOP levels were negligible (Fig. 26).

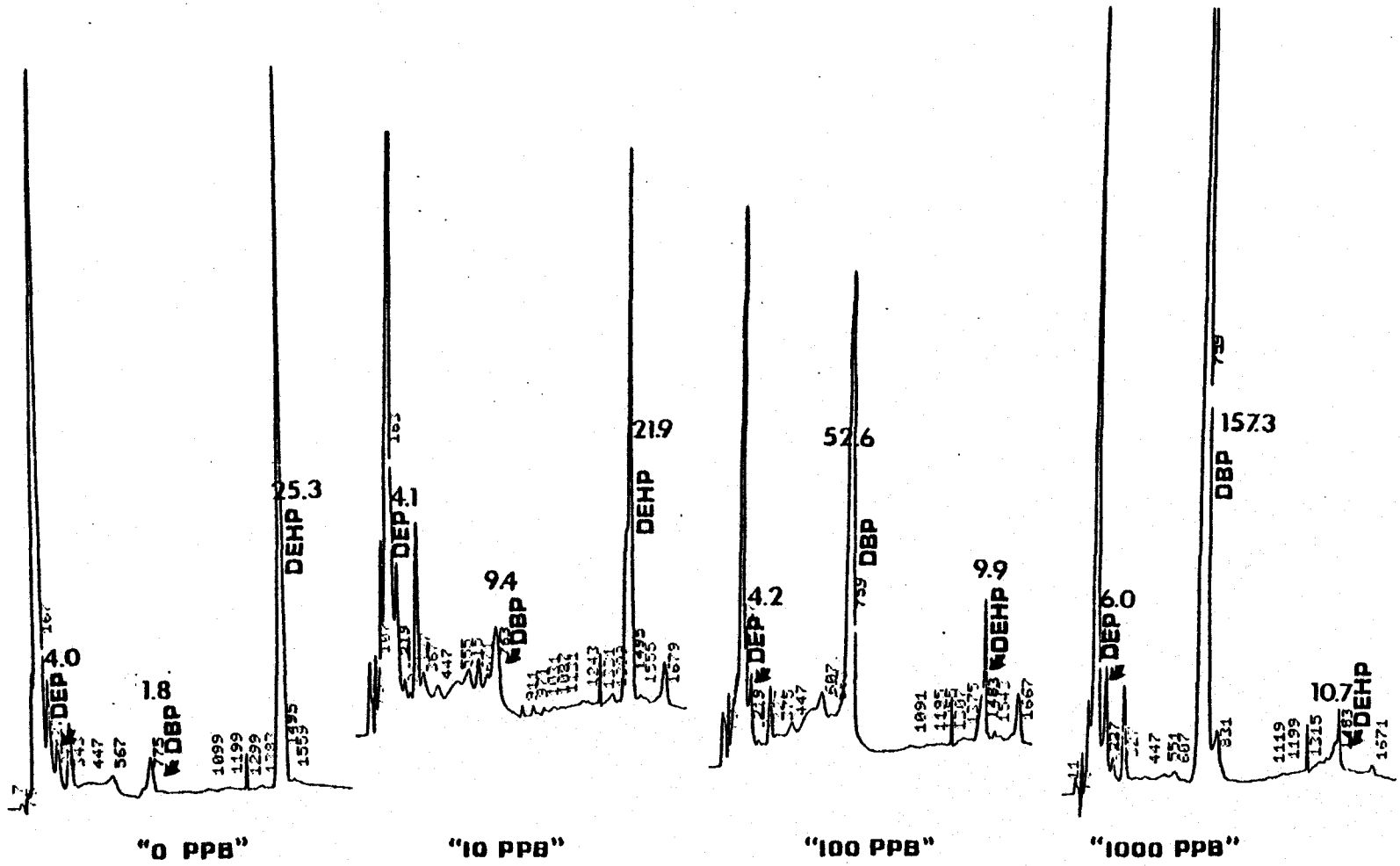


Figure 26. Phthalate content of pooled culture chamber effluent, experiment 6. Horizontal numbers indicate phthalate content in ppb, vertical numbers indicate retention time.

DISCUSSION

Preliminary Experiments

Experiments 1-4 demonstrated that although there were no consistent differences in hatching or molting success between eggs grown at 14° C and those grown at ambient temperatures, the possibility of lethal temperature fluctuations dictated that a cooling system should be used. In addition to death of the embryos, as occurred in experiment 3, temperature fluctuations approaching the limit of the tolerance can result in reduced embryo size (Patel 1959) and abnormal development (Patel and Crisp 1960). It would be preferable to grow the eggs at or near 14° C in any case since that is the temperature of the water under natural spawning conditions.

No relationship was demonstrated between lighting conditions and hatching or molting success although an unexplained failure did occur with the 4B (cold/light) group. This was puzzling in light of the success of the 4A group which came from the same adults, and the absence of any light-related effects in any of the other experiments. Apparently a few 4B eggs hatched long before the others did, resulting in an extremely low hatching index after three days.

The low-light conditions were used in subsequent experiments because it was felt they more accurately duplicated conditions inside the mantle cavity of the adult.

No problems were encountered by culturing eggs and larvae without antibiotics. Use of stage I-II molts as the last event

observed eliminates the need for feeding the larvae and was probably responsible for reducing the need for an antibiotic. However, other studies in which the larvae were not fed required antibiotics (Abbott 1975, Mix et al. 1979). Attention to maintaining near-sterile conditions was probably also a factor in the success of the present studies.

DBP Experiments

Experiment 6 showed that within-treatment variances can be greatly reduced by more precisely identifying the time of hatching to within an eight-hour period. The results also demonstrated that, when measured more accurately from the time of hatching, 72 hours was not sufficient time to allow for development of a reasonable number of stage II larvae in the control cultures. That was indicated by the hatching times recorded in experiment 6 (Table XI). Over half (18 out of 32) of the egg mass quarters were observed to have begun hatching at 12 AM, none at 8 AM, and 14 at 4 PM. If the examination and fixing schedule of previous experiments had been employed here, the eggs would have been examined once daily around 4 PM and fixed three days after hatching started. In that case, the 18 that had started to hatch by midnight would actually have gone 86 hours before fixing rather than 72, while those that had begun hatching by 4 PM would have gone about 72 hours. That would account both for the larger variances observed in some of the previous experiments and the greater mean hatching and molting indices.

TABLE XI. HATCHING PATTERNS, EXPERIMENT 6.

Day first hatching observed	Time first hatching observed		
	12 am	8 am	4 pm
5/24	-	-	6D1
5/25	6B1 6C1 6D2, 6D7	-	6A8 6B2, 6B3, 6B4 6D4
5/26	6A1 6B7, 6B8 6C2, 6C4 6D3, 6D8	-	-
5/27	6A6	-	6A2, 6A3, 6A4, 6A7 6C3
5/28	6C6 6D5	-	6A5 6B5, 6B6
5/29	6C5 6D6	-	-
5/30	6C7, 6C8	-	-

A significant effect upon molting success was seen in experiment 5 with exposure to 1000 ppb DBP; but, without confirming results from experiment 6, additional experiments upon young egg masses are required.

Overall Success of Groups not Exposed to DBP

The range of mean hatching indices in experiments 1-4 and the controls from 5 and 6 was 53.6-90.37 with a mean of 75.50. The range of molting indices was 4.19-41.98.

Although Standard Methods for Analysis of Water and Wastewater (APHA, AWWA, WPCF 1976) recommends not using results from bioassays where more than 10% of the controls die or show the effect in question, they also recognize that it is difficult to keep losses within this range when using larvae of marine crustaceans. In any case, such a criterion has little meaning unless the natural hatching success of the organism is known.

It is difficult to compare the hatching and molting indices in these experiments with other studies on in vitro culturing of Pollicipes because of differences in experimental design. Lewis (1975a) was attempting to raise larvae to the most advanced stage possible and her data were expressed as the latest stage reached by larvae under a given set of conditions. Abbott (1975) fixed cultures "when hatching was completed" [later specified as 35 days after collection (Abbott and Mix 1979)]. The mean hatching index of the controls in that experiment was 67.80 and the mean molting index was 42.22. In a later study (Mix et al. 1979) cultures were fixed at 10,

13, and 15 days after collection (bright orange egg masses were specified in this study). Mean hatching indices of the two groups of 15-day cultures were 49.6 and 57.31; molting indices were 37.62 and 43.79. It was not clear in either of those studies how pieces of intact egg mass were handled if such were present.

Hatching indices achieved in this study compare favorably with those from others. Overall molting indices were low; however, the very large variance in the range of molting indices and the results of experiment 6 indicate that they could be improved by allowing more time between first hatch and fixing.

Background Phthalate Levels

The results indicate that, although DOP can be reduced to an acceptable (< 1 ppb) level simply by preparing the seawater in glass, there is persistent contamination by DBP, DEHP, and DEP. DEP and DBP background levels can be reduced by treatment with activated charcoal, but that seemed to have no effect on DEHP levels, which fluctuated from 6.7-25.8 ppb in the charcoal treated samples.

The high levels of DEHP and low levels of other phthalates in the charcoal-treated samples may have been caused by DEHP contamination in the charcoal or overloading of the column. Specific tests on the column would be necessary to confirm or rule it out.

General phthalate contamination in the sea salts could come from the plastic bags they are packaged in, or from any plastics

(pipe or containers) used in their manufacture. The implications of these results are obvious for experimenters who use synthetic sea salts in preparing seawater.

Phthalate Levels After 24 Hours

Very little DBP was found in the control (0 ppb DBP added) seawater after 24 hours in the growth chambers, indicating that the use of a plastic cover to produce desired lighting conditions was not a significant route of contamination.

The percentage of DBP lost from solution increased with increasing initial concentration. As the DBP reached its aqueous solubility limit (about 50 ppm) it could become more prone to evaporative losses. Other routes of DBP loss could include adsorption onto the glass culture chambers or partitioning into the lipid-filled egg mass. To determine the extent of the latter, an experiment similar to 5 and 6 could be employed; when the egg masses show the first signs of hatching they could be extracted and analyzed for DBP. Dibutyl phthalate loss through degradation was probably minimal under the temperature, pH and lighting conditions used in these experiments.

In a similar bioassay using grass shrimp larvae (Laughlin et al. 1978) no DBP was detected after 24 hours from nominal concentrations of 100 and 1000 ppb. The difference in loss may be due to the use of 8 cm finger bowls, presumably uncovered, as growth chambers in that experiment. These containers would have had a larger surface area exposed to the air and would probably experience greater evaporative

losses.

Suitability of P. polymerus for Use in Invertebrate Bioassays

The use of P. polymerus as a bioassay organism would help to fill a major gap—the complete absence of procedures for cirriped crustaceans—in existing marine bioassay procedures. It is a common invertebrate on the west coast of North America and eggs can be easily collected five to six months out of the year; in addition, laboratory spawning appears possible. Color changes in developing eggs allow one to select egg masses containing desired developmental stages.

The two egg masses from each adult can be divided up into smaller units so that several experimental treatments can be performed on offspring from a single adult. The compactness of the egg masses allow use of the eggs from a number of different adults in order to compensate for differences in reproductive success between adults.

Since the eggs normally develop inside the mantle cavity of the adult and are only exposed to fresh seawater when the adults are feeding, it is easier to duplicate natural conditions in the laboratory than for other marine bioassay species such as shrimp or copepods. If the experiments are terminated after the stage I-II molt, it would not be necessary to feed the larvae, and would result in a simpler experiment less vulnerable to contamination.

In addition, a body of literature is accumulating to indicate what sort of hatching and molting success can be expected from

P. polymerus raised in the laboratory, and under what conditions they should be raised.

Recommended Design for Pollicipes Bioassays

A recommended design for Pollicipes larvae bioassays is summarized in Table XII.

Because of differences in hatching and molting between adults and the difficulty of collecting egg masses from different adults that spawned at exactly the same time, it seems advisable to use an end point based on the time when hatching is first observed rather than a given number of days after collection. Since the eggs do not all hatch at once, and some never hatch at all, some objective criteria must be used.

It is very important that an objective criterion be used to determine the start of hatching (e.g., the presence of five or more active nauplii), to check for hatching at least every eight hours once it is apparent by color that the eggs are ready to hatch, and to allow sufficient time after hatching starts for a reasonable number of the controls to molt to stage II. Eighty-six to ninety-six hours post-hatching would appear to be adequate.

The subsampling and counting procedures used here are convenient and reproducible. Treatment with trypsin is a quick and easy method of breaking up any pieces of intact egg mass without harming the eggs.

I feel that the methodology developed herein could provide a starting point for a number of useful bioassay studies with Pollicipes polymerus eggs and larvae.

TABLE XII. RECOMMENDED CULTURE CONDITIONS AND DESIGN FOR *POLLICIPES* BIOASSAYS.

Culture apparatus	As specified in text; Fig. 9.
Temperature	14° C or slightly above for eggs collected north of Point Conception, CA.
Lighting	Alternating dark/very low light cycle.
Eggs	Use young eggs (white- or light orange) for longest exposure to test substance. Cut into quarters or smaller pieces if desired.
End point	86-96 hours post-hatching; "hatching" defined as when at least five active nauplii are visible.
Counting	Undissociated egg masses should be separated with trypsin after fixing. Subsample with Stemple pipette; 500 organisms per ml optimal for counting.

SUMMARY

In this study, an *in vitro* system for culturing the eggs and larvae of the gooseneck barnacle, *Pollicipes polymerus*, was developed and tested in two bioassay experiments using the phthalate ester plasticizer dibutyl phthalate. The culture system employed aerated culture chambers composed of chemically inert substances and designed to be inexpensive, convenient to use, and to minimize external contamination of the cultures.

Pollicipes eggs were grown in synthetic seawater at 14° ± 1° C under lighting conditions of alternating darkness and very low light. Water was changed every 24 hours. Antibiotics were not necessary if prescribed methods were employed. An overall mean hatching success index of 75.5 was achieved with egg masses not exposed to dibutyl phthalate (DBP). Variances in molting success were reduced by checking the eggs for hatching every eight hours instead of every 24, and basing the end point of the experiments on the more carefully defined hatching time. Experiments indicated that allowing 86-96 hours after the first hatch before ending the experiment resulted in sufficient stage II larvae to be able to draw conclusions regarding molting success.

One of the DBP experiments showed there was a significant decrease in molting success in larvae from eggs exposed to 1000 ppb DBP from a week prior to first hatching to the end of the experiment.

Dibutyl phthalate stayed in solution between water changes to a greater degree in this system than in a system using glass finger

bowls to culture larval grass shrimp which also employed 24-hour water changes.

Several phthalate esters were found to be persistent contaminants in synthetic sea salts.

The results of this work can be used in the design of future bioassay experiments employing P. polymerus.

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