

AN ABSTRACT OF THE THESIS OF

Enrique Carrillo Barrios-Gómez for the Master of Science
(Name of student) (Degree)

in Oceanography presented on August 27, 1973
(Major Dept.) (Date)

Title: Experimental Interbreeding between Atlantic and Pacific Populations of the Marine Calanoid Copepod Acartia clausi Giesbrecht

Abstract approved Redacted for privacy
Charles B. Miller

An attempt has been made to experimentally interbreed Atlantic and Pacific populations of the marine calanoid copepod Acartia clausi Giesbrecht. Breeding between individuals from each population produced many successive generations in the laboratory, but interbreeding between populations failed to produce viable offspring. This result is strong evidence that the Atlantic and Pacific populations of A. clausi have diverged enough to become reproductively isolated. Assignment of specific rank to differentiate both populations is suggested.

Some morphological and ecological differences between Atlantic and Pacific populations of A. clausi were observed. Further studies are proposed to evaluate the constancy of these differences, and their possible use for the recognition of Pacific and Atlantic A. clausi.

Observations are presented on the general problems of copepod culture work.

Experimental Interbreeding between Atlantic and
Pacific Populations of the Marine Calanoid
Copepod Acartia clausi Giesbrecht

by

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A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

June 1974

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Date thesis is presented August 27, 1973

Typed by Suelynn Williams for Enrique Carrillo Barrios-Gomez

With love, respect and everlasting gratitude to all those who have given their love, devotion and self-sacrifice, and have given me all I have, and made possible all I am, or will be. My mother, my grandmother, and my great grandmother.

Enriqueta Barrios-Gómez Muza
Josefina Muza Barrios-Gómez
María Chuaro Muza

To my wonderful family. With their love, patience and encouragement they have made sad and difficult moments pass unnoticed. They fulfill my life with everyday joy, they are the driving force leading my actions. I love them and thank them. My wife and children.

Socorro Regino de Carrillo
Enrique Alejandro Carrillo-Regino
Paulo Alfonso Carrillo Regino
María Gabriela Guadalupe Carrillo Regino

ACKNOWLEDGMENTS

I thank my major professor Dr. Charles B. Miller for his valuable help throughout my graduate work. His time, guidance, suggestions and personal assistance during the last part of my work will be especially remembered. I also want to express my thanks to the "Consejo Nacional de Ciencia y Tecnologia" of Mexico for their support while working on my M. S. program. The original idea and initial work for this thesis came from Dr. Steve Zimmerman. I thank him.

I find no words to express my thanks to Bruce Wyatt, my friend. His trust and confidence in me, his friendly advice and encouragement during difficult times will be always remembered. I thank Dr. P. Kilho Park for his friendship, his help and his personal influence in many aspects of my academic life. To Dr. Andrew G. Carey, I thank sincerely for his time and valuable suggestions in my work. I thank Dr. John V. Byrne for continually extending to me the resources of the department. To Drs. Park, Carey, Byrne and to Bruce Wyatt I owe my present opportunity to continue my graduate work. I am very grateful to them. Without the time and effort that Dr. Peter H. Wiebe from Woods Hole Institution of Oceanography spent in collecting, sorting and sending Atlantic copepods, this work would not have been possible.

Special thanks to all those who in many ways are responsible for the completion of my degree. William T. Petersen and Christopher

J. Marlowe. Discussing many aspects of my work with them has been one of the best assets I have ever had. I owe to them in many ways my own understanding of this work. Dr. Ravenna Ukeles from the National Marine Fisheries Services at Milford, Connecticut, has provided me with phytoplankton cultures ever since I began my undergraduate work in Marine Sciences in Mexico. Thanks also to Joan Flynn for her help in teaching me the morphology of Acartia clausi, to Craig Wiese and Barbara Sullivan for their time and help when I needed it. Thanks also to the "peanut butter" people in the phytoplankton group. Their concessions for a long list of materials are sincerely appreciated.

I also want to thank Suelynn Williams for her time in typing all the drafts and final version of this thesis.

TABLE OF CONTENTS

| | |
|--|----|
| I. INTRODUCTION | 1 |
| II. LITERATURE REVIEW | 4 |
| Copepod Culture | 4 |
| Temperature | 5 |
| Food | 6 |
| Bacteria | 8 |
| Other Factors | 10 |
| III. METHODS AND MATERIALS | 14 |
| Sampling Procedure | 14 |
| Laboratory Culture | 14 |
| Interbreeding Experiment | 17 |
| IV. RESULTS | 18 |
| Interbreeding Experiment | 18 |
| Copepod Culture (General Observations) | 21 |
| Handling | 25 |
| Temperature | 26 |
| Food | 28 |
| Bacteria | 30 |
| Other Factors | 30 |
| Morphology | 31 |
| V. DISCUSSION AND CONCLUSIONS | 36 |
| BIBLIOGRAPHY | 44 |

LIST OF FIGURES

| <u>Figure</u> | | <u>Page</u> |
|---------------|--|-------------|
| 1 | <u>Acartia clausi</u> : a) Fifth leg Atlantic male; b) Adult male Atlantic. Dorsal view; c) Adult male Pacific. Dorsal view; d) Fifth leg Pacific male. | 33 |
| 2 | <u>Acartia clausi</u> : a) Fifth leg Atlantic female; b) Adult female Atlantic. Dorsal view; c) Adult female Pacific. Dorsal view; d) Fifth leg Pacific female. | 34 |

LIST OF TABLES

| <u>Table</u> | | <u>Page</u> |
|--------------|---|-------------|
| I | Summary of interbreeding attempts between Atlantic and Pacific populations of <u>A. clausi</u> . A = Atlantic, P = Pacific. | 19 |
| II | Summary of control beakers for Atlantic and Pacific populations of <u>A. clausi</u> . | 20 |
| III | Summary of culture attempts for Atlantic and Pacific populations of <u>A. clausi</u> . | 23 |
| IV | Temperature change during shipment, and standing stock of Atlantic <u>A. clausi</u> at several times during the period from March 9, 1973 to May 29, 1973. | 24 |
| V | Standing stock of Pacific and Atlantic populations of <u>A. clausi</u> at several times during the period August 24, 1972 to September 20, 1972. | 27 |
| VI | Survival of an adult population of Pacific <u>A. clausi</u> influenced by temperature and shipping time. Each horizontal line represents one thermos bottle. | 29 |
| VII | Standing stock of a population of Pacific <u>A. clausi</u> as affected by water quality at several times during the period from September 22, 1972 to December 8, 1972. | 32 |
| VIII | Standing stock of a population of Pacific <u>A. clausi</u> affected by EDTA at several times during the period from September 22, 1972 to December 8, 1972. | 32 |

EXPERIMENTAL INTERBREEDING BETWEEN ATLANTIC AND
PACIFIC POPULATIONS OF THE MARINE CALANOID
COPEPOD ACARTIA CLAUSI GIESBRECHT

I. INTRODUCTION

The nomenclature of organisms is primarily based on morphological differences as the decisive criteria for assignment of species rank. Classification of species in the marine environment is not an exception. Systematicists have questioned the validity of the morphological approach for a long time: Simpson (1951) warned against a classification based strictly on degrees of morphological differences between organisms, because these differences are frequently greater within a species than between different species. Mayr (1963) also stressed the weakness of a purely morphological species concept; he based his argument on the presence of intraspecific variation within a single population or between conspecific populations and on the existence of sibling species. He concluded that: (1) the decisive criterion for assignment of species status should be the reproductive isolation of populations; (2) populations sharing a common gene pool are termed conspecific; (3) definitions stressing these biological characteristics (1 and 2) can be referred to as biological species definitions, and (4) morphological criteria, when applied by the taxonomist should be used as secondary indicators of reproductive isolation.

The degree to which marine animal species described on morphological grounds fall within the biological species definition has been difficult to evaluate. Assessment of reproductive isolation by interbreeding experiments has been greatly hampered by the difficulty of culture techniques for these animals. Little work has been done on interbreeding allopatric populations of conspecific copepods, though they are the most abundant group of animals in the marine environment. An exception is the work done with allopatric populations of nearshore harpacticoid copepods by Battaglia (1956) and Bozic' (1960) (both cited by Mayr, 1963). A reduction of fertility was found in some crosses between Tisbe reticulata Bocquet from Brittany and from the Venetian lagoon and between various populations of Tigriopus fulvus.

Studies on natural and experimental interbreeding in other crustacea have been done more frequently. For instance, Lecher (1967), Prunus (1968), and Solignac (1969) studied the genetic factors affecting interbreeding in different populations of the isopod Jaera albifrons. Bocquet and Solignac (1969) studied the morphological features of experimental hybrids between two species of Jaera. Shaw and Frey (1968), working with a chydorid cladoceran and Halfer-Cervini et al. (1968), working with Artemia, discussed the assignment of species ranks to populations on the basis of hybrid

inviability and differences in chromosome number. Stock (1969) discussed the interbreeding of two species of Gammarus.

This thesis reports some interbreeding experiments between allopatric, conspecific populations of the marine calanoid copepod Acartia clausi Giesbrecht. An attempt is made to evaluate and compare the relevance of the morphological and biological species concepts for species assignment in this group of marine organisms. East and West Coast populations of A. clausi were chosen as experimental organisms because of their broad distribution. For instance, Subbaraju (1967) states that copepods belonging to the genus Acartia are found widely distributed in all the seas, where 34 species belonging to eight subgenera are known. Esterly (1924), Wilson (1932), Rose (1933), and Conover (1956), report A. clausi in estuarine waters over much of the world. A. clausi was also chosen because its development and biology have been studied extensively by Conover (1956), among others, and because the difficulties associated with its culture for several generations under laboratory conditions have been overcome by Zillioux (1969).

II. LITERATURE REVIEW

Copepod Culture

The importance of keeping large populations of marine copepods in the laboratory has been stressed by a number of workers. Crawshay (1915), Raymont and Gross (1942), Conover (1967), and Corkett and Urry (1968) commented on the utility of culturing copepods for the confirmation of field observations on their biology, reproduction, and life cycles. Shiraishi and Provasoli (1959), Neunes and Pongolini (1965), Lewis (1967), and Zillioux (1969) stressed the importance of copepod culture for detailed physiological and toxicological analyses and for studies on the nutritional requirements of different species. Urry (1965), Zillioux and Wilson (1966), and Katona and Moodie (1969) discussed the importance of marine calanoid copepods in the food web of the oceans and the advantages of their maintenance in the laboratory for obtaining a better understanding of their trophic relationships.

Interest in the maintenance of large populations of copepods in the laboratory, however, is not new. Crawshay (1915) discussed some of the early difficulties found in the culture of marine calanoid copepods and speculated upon the importance that certain factors might have in future work.

Since then, for a long period of time calanoid copepods were regarded as being almost impossible to culture with success. It has

been only in the last few years that most of the difficulties associated with their rearing have been overcome, and several species have been successfully cultured in the laboratory for several generations under a broad spectrum of environmental conditions (Raymont and Gross, 1942; Zillioux and Wilson, 1966; Mullin and Brooks, 1967; Heinle, 1969; Katona and Moodie, 1969).

Several factors have been considered by different authors to be of primary importance to the success of their rearing experiments. Most of them agree that minimal handling and disturbance of the organisms, coupled with temperature and bacterial control and an adequate food supply are probably the most important factors.

Temperature

The importance of temperature in the success of laboratory culture of marine copepods has been stressed by several authors. Crawshay (1915) noted the adverse effects of temperature fluctuations and regarded this as one of the probable factors causing the failure of earlier experiments. Raymont and Gross (1942), rearing Calanus finmarchicus, concluded also that constant temperatures in their cultures are important. They suggested that gradual increments could be tolerated by adult copepods, but that naupliar and early copepodite stages were less tolerant to even slight changes of temperature. Corkett and Urry (1968) rearing Pseudocalanus elongatus

Boeck found an inverse correlation between temperature and survival; they concluded that the greater survival of the copepod at low temperatures was caused by a lowered metabolic rate.

On the other hand, Corkett (1967) concluded that this factor was not critical in laboratory cultures on the basis of his success rearing Pseudocalanus minutus Krøyer and Temora longicornis Müller at several different temperatures. He does not specify, however, whether each one of those different temperatures used was kept constant.

Food

From the early beginning the importance of suitable food during laboratory culture of copepods was stressed (Crawshay, 1915). Since then, the nutritional value of several species of phytoplankton and nanoplankton, as well as other food sources have been investigated, and their effect on the development and the survival of several species of copepods has been assessed. Raymont and Gross (1942) fed C. finmarchicus with different kinds of diatoms and autotrophic flagellates and concluded that copepods were apparently able to utilize a wide range of food. They suggested that flagellates are necessary for the survival of naupliar and copepodite stages. Urry (1965), culturing P. elongatus, utilized the chryomonad Isochrysis galbana Parke as a standard food. He concluded (citing Davis and Guillard, 1958; and

Provasoli et al., 1959) that it appeared to be an ideal food for several herbivores with no evidence of toxicity even at high concentrations. On the other hand, Shiraishi and Provasoli (1959) observed that I. galbana as well as other "algal flagellates" caused larval mortality and adult infertility of Tigriopus japonicus after several generations when fed singly. On the basis of their results, they recommended the use of a mixed diet, in which bacteria or vitamins could also be included.

Following this suggestion, Lewis (1967) reduced the mortality of egg and feeding stages of the copepod Euchaeta japonica Marukawa in culture by feeding a mixed diet consisting of diatoms, flagellates and an enrichment solution made of dissolved organics, vitamins, and metals. Zillioux and Wilson (1966) and Zillioux (1969) cultured Acartia tonsa Dana, and A. clausi on a diet consisting of equal parts of Rhodomonas sp. and I. galbana.

Some authors apparently ignored the total density of food added to their cultures. For instance, Shiraishi and Provasoli (1959), with T. japonicus, Neunes and Pongolini (1965), with Euterpina acutifrons Dana, and Barr (1968), with Tisbe furcata Baird, among others, did not measure the amount of food utilized. They probably assumed that the food added would be more abundant than that found by the copepods in their natural environment (Raymont and Gross, 1942).

However, the importance of feeding an adequate food concentration

is obvious, since according to Zillioux and Wilson (1966) the development time is lengthened in laboratory populations fed at suboptimal levels. Also, bacterial contamination usually occurs in cultures fed beyond optimum levels (Zillioux, 1969). Either problem may be present when the concentration of food added is unknown. When the density of the food is known, ranges of the total food concentration added to the cultures are very high. Zillioux and Wilson (1966) culturing A. tonsa utilized a total food density as low as 10,000 cells/ml, whereas Corkett (1967), rearing T. longicornis and P. minutus used densities as high as 300,000 cells/ml.

For A. clausi, Zillioux (1969) determined that optimum total food concentrations are around 100,000 cells/ml of a mixture containing equal parts of R. baltica and I. galbana.

Bacteria

The effect that bacteria may have in the culture of marine copepods has been a matter of concern for over 50 years. As an example of the different control measures introduced, Crawshay (1915) experimentally introduced the ciliate Euplotes sp. and successfully controlled several kinds of bacteria. More than 50 years later, Zillioux (1969) controlled bacteria with the introduction of E. vannus Möller in his cultures of A. clausi and A. tonsa.

By a different approach, Neunes and Pongolini (1965), rearing

E. acutifrons, alternately used penicillin and streptomycin at a concentration of 6.5 mg/l. They concluded, on the basis of experimental evidence, that the bacteriostatic effect of these antibiotics was short, lasting less than a day. Therefore they suggested the utilization of antibiotics only during the initial period of copepod culture when "the organisms are probably in a weakened condition after having undergone the straining procedure of collection." Conover (1967) culturing Calanus hyperboreus Krøyer, also used penicillin and streptomycin alternately, but in a concentration of 50 mg/l. This concentration is considered by Neunes and Pongolini (1965) to be high and detrimental to the "vitality of the copepods". Corkett and Urry (1968), following the suggestion of Cviic (1953) (cited by Corkett and Urry, 1968) that penicillin and streptomycin are more effective when used together, combined both antibiotics and concluded that survival of P. elongatus was best at a concentration of 10 i. u. /ml of each.

To control excessive growth of bacteria, Raymont and Gross (1942) changed their cultures to clean dishes and clean water whenever an appreciable growth of bacteria was evident. Urry (1965), rearing P. elongatus, avoided contamination by transferring copepods to clean dishes and clean water once every week. Corkett and Urry (1968), also rearing P. elongatus, suggested that the combined use of antibiotics and the transfer of copepods to fresh medium and clean dishes at intervals of one week or less would keep contamination low.

Jacobs (1961), rearing Pseudodiaptomus coronatus Williams, sterilized all beakers before use, and Heinle (1969) reported an apparent lack of bacterial contamination in his cultures of A. tonsa and Eurytemora affinis Poppe with the use of artificial sea water.

Other Factors

Other factors have also been regarded as having more or less influence in the culture of marine copepods. Certain authors consider the number of organisms per volume of water and the absolute size of the container to be important. Others include optimum oxygen levels, illumination, and quality of the water used.

Urry (1965) assigned maximum densities for P. elongatus at not more than three copepods per 100 ml of water. Corkett and Urry (1968) reviewed the densities used by other authors and concluded that for P. elongatus the survival was not affected by densities as high as one copepod per 10 ml of water, but in practice they used three copepods per 110 ml of water. Zillioux and Wilson (1966) observed that cultures of A. tonsa with 100 nauplii or more per liter "would usually show retarded or erratic development, followed by high mortality." On the other hand, Neunes and Pongolini (1965) kept E. acutifrons at densities as high as 300 copepods per 100 ml of water. Barr (1968) kept approximately from 100-200 T. furcata at all stages of development in 100 ml of water, and Zillioux (1969) kept A. tonsa

and A. clausi at densities as high as 40 copepods per 100 ml of water, all of them, apparently with success.

As for the size of the container, Zillioux and Wilson (1966), culturing A. tonsa commented that there appeared to be an increase in mortality with a decrease in dish size. Corkett (1967), suggested that mortality in P. minutus could be decreased with an increase in the volume to surface ratio in the containers. Katona and Moodie (1969), working with P. elongatus found greater mortality in small vessels. They suggested that it was caused by a large surface to volume ratio, which encouraged growth of bacteria and entrapment of nauplii at the surface of the water.

Crawshay (1915) concluded that apparently no advantage was found in aerating the water in the cultures if the water was naturally aerated at the outset. Zillioux (1969), however, had almost complete mortality in one of his cultures containing A. clausi, and attributed this to a very low O₂ concentration. Dissolved O₂ is now maintained in his cultures by aeration around 9.5 to 9.8 ppm.

Almost all workers have illuminated their cultures at various intensities. However, Urry (1965) and Corkett and Urry (1968) culturing P. elongatus, maintained their cultures in the dark. Zillioux and Wilson (1966), rearing A. tonsa, commented that research on the subject is incomplete, but that light appears to have no effect on either development or survival; and Corkett (1967), rearing P. minutus,

concluded on the basis of experimental evidence that light is not a critical factor.

Several authors have been concerned with the importance of water quality in the success of their cultures. Crawshay (1915), culturing several kinds of marine organisms, used sea water collected from different places and treated in several ways. Neunes and Pongolini (1965), rearing E. acutifrons, found that the survival time of mass cultures of zooplankton was shorter for water sterilized (autoclaved for 20 min at 110°C) than for non-sterilized sea water. They concluded that this was probably due to "chemical changes effected by the high autoclaving temperature." Corkett (1967), rearing P. minutus and T. longicornis, concluded that success of his laboratory culture was probably due, among other factors, to the "use of fresh sea water taken from the same area of the sea as the adults." Heinle (1969) concluded that sea water, particularly that of neritic origin, has disadvantages as a culture medium, and introduced the use of artificial sea water. He reared with success A. tonsa but was unable to do the same with A. clausi under the same conditions. Zillioux (1969) failed to rear A. clausi and A. tonsa using the same artificial salts as Heinle (1969). He succeeded in his attempts using another formula fitted better for the higher salinities used in his experiments.

Even though the methods and kinds of water utilized in the

culture of marine organisms may differ in several ways, a point in which several authors seem to coincide is in the use of filtered water (Urry, 1965; Zillioux and Wilson, 1966; Conover, 1967; Barr, 1968; Corkett and Urry, 1968; Katona and Moodie, 1969).

III. METHODS AND MATERIALS

Sampling Procedure

All the copepods used in this work were collected at Yaquina Bay, Oregon in the Pacific, and at Woods Hole, Massachusetts in the Atlantic. They were captured with 1/2 m and 1 m nets equipped with mesh of 0.239 mm or finer. Pacific collections were made on five occasions from June 1971 to February 1973; Atlantic collections were made on four occasions, from September 1972 to June 1973.

Upon capture at Yaquina Bay, the plankton sample was strained through a coarse nylon mesh to remove large animals, and placed in several thermos jars. At Woods Hole, A. clausi were sorted from the plankton by pipette and placed in 1 liter thermos jars, or large 6 liter styrofoam jars, in a concentration of about 100-150 individuals per jar. The jars were partially full of sea water containing 37 mg/l (10^{-4} mol) of EDTA, a complexing agent, and 6.25 mg/l of Penicillin G, an antibiotic used only during the initial period of culture (Neunes and Pongolini, 1965). The thermos jars from Woods Hole were shipped via air freight, with a transit time of 1-3 days.

Laboratory Culture

Upon arrival at the laboratory in Corvallis, Oregon the plankton samples were diluted with pre-cooled sea water, and adult A. clausi

were sorted into different containers. A concentration of not more than one copepod per 35 ml of water was allowed, following Urry (1965) and Corkett and Urry (1968).

After the initial sorting of adult copepods, individuals at all levels of development were transferred to clean dishes and clean sea water regularly every week to avoid excessive growth of bacteria (Urry, 1965; Corkett and Urry, 1968). Most of the water from each container was drained by means of a siphon modified to avoid excessive speed in the flow of water. The siphon was fitted with nylon gauze at the intake to prevent any loss of organisms. Water was carefully poured into a Petri dish and examined under a dissecting microscope. A dropper with an adequate aperture to avoid damage to the animals was used to transfer them into clean beakers. Adult males, females, copepodites, and naupliar stages were kept in 650 ml beakers, with densities of up to 10 adult copepods, 25 copepodites, or 100 nauplii per 500 ml of water. Copepodites for breeding experiments were reared individually in 150 ml beakers until adult stage was reached, allowing approximately 100 ml of water per animal. This isolation prevented contact of males and females prior to the experiment. All the containers were covered with a "Speedyvap" watchglass cover designed to permit free exchange of oxygen.

Pure algal cultures used as food were kept in the same cold room with the copepods in 2800 ml Fernback flasks covered with

aluminum foil, with approximately 2 liters of culture per flask. Nutrients and vitamins were prepared and added following a technique described by Curl (MS.). Temperature was kept constant at approximately $17 \pm 0.5^\circ\text{C}$, and constant illumination was provided by means of four 40 watt cool white lamps suspended above the flasks. Algal densities were determined by counting samples on haemocytometer slides (Fuchs-Rosenthal ruling) as described by Urry (1965) and Corkett and Urry (1968). A food mixture consisting of the diatom Thalassiosira nordenskioldii Gravidia in a concentration of approximately 5,000 cells/ml, the flagellate Rhodomonas sp., 30,000 cells/ml, and the chrysomonad I. galbana 40,000 cells/ml, was fed to the organisms at the time of each transfer. Thereafter, enough food was added every 3-4 days to maintain the initial concentration.

Pacific sea water containing EDTA in a concentration of 37 mg/l (Neunes and Pongolini, 1965) was used in all the experiments. It was collected either in the Pacific Ocean within a few miles of the coast, or in Yaquina Bay, at high tide. The water was membrane filtered twice before use.

Glassware was washed with distilled water and sterilized before use (Jacobs, 1961), avoiding the use of any detergent (Loosanoff and Davis, 1963; Heinle, 1969).

Interbreeding Experiment

The environmental conditions of the experiment were the same as those already described for the laboratory culture. Individually reared Atlantic females and Pacific males, as well as Pacific females and Atlantic males were placed in separate beakers. The number of organisms per beaker depended on the availability of males and females of each species, up to a maximum of 10 copepods per beaker. Control beakers containing females and males of each population were also set up at the same time as the interbreeding experiments. After one week, the beakers were examined, and the presence of eggs, nauplii, or spermatophores attached to the females was recorded.

Several individually reared females of both Atlantic and Pacific Ocean species were kept singly in 150 ml beakers to determine production of eggs by unfertilized females as observed by Jacobs (1961) and Corkett (1967). Several adult copepods of both species were preserved in formalin and mounted on slides with glycerin for morphological comparison of important characters. Dissection was made under a binocular dissecting microscope, and the drawings were made with a camera lucida.

IV. RESULTS

Interbreeding Experiment

The interbreeding experiments with controls are summarized for the three-month period May to July 1973 in Tables I and II. Differences in the number of males and females of each population were caused by the availability of copepods at the time of each experiment. There were more experiments undertaken with Atlantic females and Pacific males than with Pacific females and Atlantic males (see Table I) owing to the difficulty of obtaining males from the Atlantic stock population as compared with the Pacific stock population.

Nauplii or females carrying spermatophores were found only on four occasions in the interbreeding experiments: On June 5th, one female was found alive carrying a spermatophore and two early nauplii were found alive in the same beaker. On June 21st, one dead female was found with one spermatophore attached. On July 24th two cases were recorded, each one in a different beaker. In the first, one female was found alive with one spermatophore attached; in the second, two dead early nauplii were found. In all cases where live nauplii or fertilized females from interocean crosses were found, they were dead within a week after the observation was made.

Most of the copepods used for these experiments came from cultures started on February 22, 1973 for the Pacific population

Table 1. Summary of interbreeding attempts between Atlantic and Pacific populations of A. clausi.
A = Atlantic, P = Pacific

| Date started | Source and No. of adults at the beginning of exp. | Date ended | Source and No. of adults alive at the end of exp. | No. of nauplii found at the end of exp. | No. of ♀ carrying a spermat. at the end of exp. |
|--------------|---|------------|---|---|---|
| May 29* | 5 A ♀ x 5 P ♂ | June 5 | 1 A ♀ 5 P ♂ | 2 | 1 |
| June 28 | 3 A ♀ x 4 P ♂ | July 5 | 3 A ♀ 4 P ♂ | 0 | 0 |
| June 28 | 3 A ♀ x 4 P ♂ | July 5 | 3 A ♀ 3 P ♂ | 0 | 0 |
| July 2 | 5 A ♀ x 4 P ♂ | July 9 | 3 A ♀ 1 P ♂ | 0 | 0 |
| July 4 | 3 A ♀ x 4 P ♂ | July 11 | 2 A ♀ 0 P ♂ | 0 | 0 |
| July 5 | 3 A ♀ x 3 P ♂ | July 12 | 2 A ♀ 0 P ♂ | 0 | 0 |
| July 11 | 4 A ♀ x 3 P ♂ | July 18 | 2 A ♀ 1 P ♂ | 0 | 0 |
| July 17 | 6 A ♀ x 4 P ♂ | July 24 | 5 A ♀ 4 P ♂ | 0 | 1 |
| July 17 | 6 A ♀ x 5 P ♂ | July 24 | 6 A ♀ 4 P ♂ | 0 | 0 |
| July 17 | 6 A ♀ x 1 P ♂ | July 24 | 6 A ♀ 1 P ♂ | 0 | 0 |
| July 17 | 2 A ♀ x 3 P ♂ | July 24 | 2 A ♀ 1 P ♂ | 2 | 0 |
| June 14 | 6 P ♀ x 6 A ♂ | June 21 | 5 P ♀ 4 A ♂ | 0 | 1 |
| June 14 | 6 P ♀ x 6 A ♂ | June 21 | 5 P ♀ 4 A ♂ | 0 | 0 |
| July 17 | 5 P ♀ x 1 A ♂ | July 24 | 2 P ♀ 1 A ♂ | 0 | 0 |
| July 23 | 4 P ♀ x 3 A ♂ | July 30 | 4 P ♀ 2 A ♂ | 0 | 0 |
| July 23 | 4 P ♀ x 3 A ♂ | July 30 | 4 P ♀ 2 A ♂ | 0 | 0 |

* Atlantic females obtained from March 9, 1973 culture (not individually reared).

Table II. Summary of control beakers for Atlantic and Pacific populations of A. clausi.

| Date started | Kind and No. of adults at the beginning of exp. | Date ended | Kind and No. of adults alive at the end of exp. | No. of nauplii found alive at the end of exp. |
|--------------|---|------------|---|---|
| June 28 | 3 ♀ x 4 ♂ AT | July 4 | 3 ♀ 2 ♂ | 71 |
| June 28 | 3 ♀ x 4 ♂ AT | July 4 | 1 ♀ 1 ♂ | 88 |
| July 2 | 6 ♀ x 1 ♂ AT | July 9 | 6 ♀ 1 ♂ | 45 |
| July 4 | 3 ♀ x 2 ♂ AT | July 11 | 2 ♀ 0 ♂ | 18 |
| July 7 | 3 ♀ x 3 ♂ AT | July 14 | 3 ♀ 1 ♂ | 107 |
| July 9 | 6 ♀ x 4 ♂ AT | July 16 | 1 ♀ 3 ♂ | >25 |
| July 15 | 7 ♀ x 3 ♂ AT | July 22 | 5 ♀ 2 ♂ | >25 |
| July 16 | 7 ♀ x 3 ♂ AT | July 23 | 4 ♀ 3 ♂ | >25 |
| July 16 | 6 ♀ x 3 ♂ AT | July 23 | 2 ♀ 1 ♂ | >25 |
| July 23 | 4 ♀ x 3 ♂ AT | July 30 | 3 ♀ 2 ♂ | 55 |
| July 23 | 4 ♀ x 3 ♂ AT | July 30 | 2 ♀ 2 ♂ | 33 |
| June 28 | 3 ♀ x 4 ♂ PAC | July 4 | 1 ♀ 4 ♂ | 12 |
| June 28 | 3 ♀ x 4 ♂ PAC | July 4 | 2 ♀ 4 ♂ | 64 |
| June 14 | 6 ♀ x 6 ♂ PAC | June 21 | 5 ♀ 6 ♂ | 80 |
| June 14 | 6 ♀ x 6 ♂ PAC | June 21 | 4 ♀ 4 ♂ | 67 |
| July 3 | 7 ♀ x 5 ♂ PAC | July 10 | 3 ♀ 4 ♂ | 29 |
| July 5 | 3 ♀ x 8 ♂ PAC | July 12 | 1 ♀ 3 ♂ | 14 |
| July 23 | 4 ♀ x 3 ♂ PAC | July 30 | 3 ♀ 3 ♂ | 89 |
| July 23 | 4 ♀ x 3 ♂ PAC | July 30 | 4 ♀ 2 ♂ | 48 |

and June 6, 1973 for the Atlantic population. However, Atlantic females from a culture started on March 9, 1973 were used in the first interbreeding attempt started on May 29, 1973. These females were not individually reared, but no spermatophores or nauplii were observed for a period of two weeks before the beginning of the experiment. Unfertilized eggs were found in all beakers containing individually reared females of both populations.

In all cases there was successful breeding in the control experiments between individuals from the same population (Table II). "Success" is defined as the production of abundant offspring and the normal development of these into a fertile adult population. The number of nauplii produced ranged from 12 for a Pacific population on July 4th to greater than 107 for an Atlantic population on July 15th. On July 16th, 22nd, and 23rd, at least 25 nauplii were recorded for each beaker in the Atlantic population. Females carrying spermatophores were recorded at times for both Atlantic and Pacific copepods in the control beakers.

Copepod Culture

Summary of General Observations. The main purpose of the culture work was to maintain a healthy and abundant population of copepods for the interbreeding experiments. In general, this purpose was achieved. Interbreeding experiments were set up; an abundant

population was available at all times for several months, and no unusual contamination from bacteria was observed. A partial exception to this statement is the Atlantic population. An abundant and healthy population was generally obtained, but, as stated previously, the number of males produced was always low, therefore limiting the size of the interbreeding experiments.

Table III summarizes the attempts made to establish a laboratory population for both Atlantic and Pacific A. clausi. The cultures started on September 21, 1972 and February 22, 1973 for the Pacific, and June 6, 1973 for the Atlantic are considered successful.

Excessive handling of the copepods by frequent water changes, presence of bacteria and bottom debris, inappropriate food densities added to the cultures, large numbers of copepods per beaker, and the probable toxic effect of Chlorella sp. used as food are considered to be the most important factors affecting the failure of June 17, 1971 and July 22, 1971 cultures of A. clausi Pacific. The failure of the Pacific culture started on June 29, 1972 and the failure to start the Atlantic culture of September 8, 1972 are believed to be due to the design of a new siphon introduced into the culture methods on September 8, 1972.

A special case of failure to maintain a laboratory culture is that of March 9, 1973 for an Atlantic population (Table IV). The copepods were apparently healthy, active, not contaminated by bacteria, and

Table III. Summary of culture attempts for Atlantic and Pacific populations of A. clausi.

| Date culture was started | Place of collection | Approximate No. of weeks that cultures were maintained in the laboratory | Date culture was stopped | Reasons for stopping culture |
|--------------------------|-----------------------|--|--------------------------|--|
| June 17, 1971 | Yaquina Bay, Ore. | 4 | July 19, 1971 | Excessive handling, bacteria and bottom debris, inappropriate food, large no. of copepods per beaker, toxic effect of <u>Chlorella</u> sp. |
| July 22, 1971 | Yaquina Bay, Ore. | 4 | Aug. 24, 1971 | |
| June 29, 1972 | Yaquina Bay, Ore. | 11 | Sept. 20, 1972 | Probably mechanical |
| Sept. 21, 1972 | Yaquina Bay, Ore. | 13 | Dec. 31, 1972 | End of experiments |
| Feb. 22, 1973 | Yaquina Bay, Ore. | > 22 | ----- | Still under culture |
| Sept. 8, 1972 | Vineyard Sound, Mass. | 2 | Sept. 20, 1972 | Probably mechanical |
| Mar. 9, 1973 | Woods Hole, Mass. | 10 | May 29, 1973 | Undetermined. Most probably temperature |
| June 6, 1973 | Woods Hole, Mass. | > 8 | ----- | Still under culture |

Table IV. Temperature change during shipment, and standing stock of Atlantic A. clausi at several times during the period from March 9, 1973 to May 29, 1973.

| Date | Time needed for shipment to arrive (hrs) | Temp of water where adults were collected (°C) | Temp of water in thermos upon arrival at lab (°C) | Temp of water in lab (°C) | $\Delta t^{\circ}\text{C}$ | Total No. adults alive upon arrival |
|---------|--|--|---|---------------------------|----------------------------|-------------------------------------|
| March 9 | 72 | 2 | 13 | 17 ± 0.5 | 15 | 128 |

| Date | Total No. adults alive | Total No. copepodites alive | Total No. nauplii alive |
|----------------|------------------------|-----------------------------|-------------------------|
| March 9, 1973 | 128 | -- | --- |
| March 17, 1973 | 86 | -- | 105 |
| April 3, 1973 | 32 | 18 | 135 |
| April 25, 1973 | 5 | -- | 32 |
| May 14, 1973 | 4 | 9 | --- |
| May 24, 1973 | 5 | -- | --- |
| May 29, 1973* | 5 | -- | --- |

* All females. Used for the first attempt of interbreeding experiments.

feeding normally from the beginning to the end of the culture. However, the adult population declined steadily, apparently from low production of nauplii and an extended developmental time. A temperature change of approximately 15°C occurred in the first 72 hrs. However, no signs of detrimental effect were noticed in the behavior of the copepods. Low recruitment is considered to have been the main reason for the decline of the population, but subtle effects of temperature shock are also taken into account.

Handling

Physical or mechanical damage to the copepods as a direct result of handling was possible both at the time they were collected and delivered to the laboratory in thermos or styrofoam bottles, and at the times of their weekly transfer to clean beakers and clean water. Styrofoam bottles were suitable for shipment. Survival of copepods after delivery is comparable to that of thermos bottles, with the additional advantage that there is no breakage.

Adult copepods, copepodites and advanced naupliar stages seemed to withstand the shipping procedures with no detrimental effect; both Atlantic and Pacific populations of A. clausi have been maintained for several generations up to this date. Excessive handling of copepods in culture, however, is regarded as detrimental, and this appears to have been one of the factors causing failure to

maintain growing populations in the initial attempts at culture. In these early attempts nauplii from the parental beakers were removed as soon as an appreciable number were seen. Early stages may be more susceptible to handling, and this procedure may have resulted in low survival of these nauplii to copepodite stages and therefore to culture failure.

Temperature

Extreme changes of temperature over short periods were avoided as far as possible. Nevertheless, some occurred with no apparent mortality resulting. For instance, on August 30, 1972, a failure of the cooling system lowered the temperature in the laboratory to near 0°C overnight, almost a 17°C change. The copepods at all levels of development remained active and healthy. No unusual mortalities were recorded over a seven-day period after the failure. However, most of the copepods under culture were dead by September 20th. The temperature excursion was probably not the factor causing this mortality. During the experiment the Pacific population showed an increase in the number of copepods in all age groups (Table V). Also most of the Atlantic population died by September 20th, even though this culture was started after the temperature excursion. It is believed that mortality in both populations was caused by physical damage to the copepods which resulted from a new siphon design

Table V. Standing stock of Pacific and Atlantic populations of A. clausi at several times during the period August 24, 1972 to September 20, 1972.

| Date | Total No. adults alive | Total No. copepodites alive | Total No. nauplii alive | Population |
|-----------------|------------------------|-----------------------------|-------------------------|------------|
| August 24, 1972 | 64 | 20 | 201 | PAC |
| Sept. 7, 1972* | 75 | 51 | 467 | PAC |
| Sept. 20, 1972 | 2 | 16 | 47 | PAC |
| Sept. 8, 1972 | 98 | -- | --- | AT |
| Sept. 14, 1972 | 28 | -- | 260 | AT |
| Sept. 20, 1972 | 3 | -- | 2 | AT |

* Siphon was introduced to the cultures on September 8.

introduced on September 8th. Both populations decreased after that date (Table V).

Even with the use of thermos or styrofoam bottles, changes in temperature were usual during the shipment of copepods. An experiment was set up on September 21, 1972 to test the importance of shipping time and temperature change to survival (Table VI). Changes in temperature were high in all three cases, but survival was good and very similar. After the completion of the experiment, the remaining copepods were used to start a new culture. They were successfully cultured in the laboratory for more than two months.

Food

The total of 75,000 cells/ml maintained in the cultures seemed to provide an abundant food concentration. All age groups had stomachs full of food every time observations were made, and there were abundant fecal pellets in the bottom of the beakers. These observations showed that T. nordenskioldii spent the shortest amount of time in suspension. On the bottom it mixed with fecal pellets and other debris to form large clusters of dead cells. Most of the time, eggs and dead nauplii could be found trapped in these clusters or moving slowly surrounded by small amounts of debris. On occasion copepodites and adults were also observed under the same circumstances. This is regarded as a probable factor affecting survival in

Table VI. Survival of an adult population of Pacific A. clausi influenced by temperature and shipping time. Each horizontal line represents one thermos bottle.

| Duration of the exp. (hrs) | Temp. of water where adults were collected (°C) | Temp. of water when thermos bottle was opened (°C) | Temp. of water in the lab (°C) | Original No. of adults in thermos bottle | No. of adults alive at end of exp. |
|----------------------------|---|--|--------------------------------|--|------------------------------------|
| 24 | 11.5 | 20.8 | 17 ± 0.5 | 100 | 61 |
| 24 | 11.5 | 20.7 | 17 ± 0.5 | 100 | 69 |
| 48 | 11.5 | 18.4 | 17 ± 0.5 | 100 | 69 |

the cultures, particularly of nauplii.

Bacteria

No major bacterial problem was seen through the duration of the culture attempts. No record of unusual mortalities due to bacterial contamination was kept. Only on a few occasions (usually when cultures were overfed) were some adults seen with a film on the urosome, presumably bacteria. Copepodites were seldom seen with any similar film, and no nauplii were seen under these circumstances. Unidentified ciliate protozoans, on the other hand, were more commonly seen in the bottom of the beakers. Their presence was usually, but not necessarily, correlated with an overabundant supply of food. Scattered growths of an unidentified green alga were also observed on occasions in the walls of some beakers, and usually disappeared when water was changed. An observation of two harpacticoid copepods was made on July 11, 1973, and constitutes the only observation of copepods other than A. clausi in the cultures.

Other Factors

No explicit experiments were arranged to determine the effect of density or size of the containers on the survival of copepods. However, it was noticed that survival was lower as the density of copepods per beaker increased or the size of the beakers decreased.

Water from a wide range of different places of origin was used indiscriminately throughout the culturing period. Apparently, success is independent of the kind of water used, but depends largely on the presence or absence of EDTA. An experiment was set up with a Pacific population of *A. clausi* to test the effect of autoclaved water and EDTA in the cultures (Tables VII and VIII). It can be seen from Table VII that apparently copepods reared with autoclaved water and EDTA developed and survived as well as those cultured with non-autoclaved water and EDTA. Both cultures were maintained successfully for more than two months in the laboratory until they were stopped at the end of the experiment. On the other hand, as seen in Table VIII, the copepods cultured without EDTA added to the water were dead approximately one month after the cultures were started. All four cultures were started under the same conditions, differing only in the type of water used, and in the presence or absence of EDTA in the water.

Morphology

Figures 1 and 2 show a gross morphological comparison between males and females from each population. Fifth legs of both sexes are also compared. Size seems to be a strong morphological feature distinguishing both populations. Atlantic males and females are bigger than Pacific males and females. Also, there is a much

Table VII. Standing stock of a population of Pacific A. clausi as affected by water quality at several times during the period from September 22, 1972 to December 8, 1972.

| Date | Not Autoclaved water + EDTA | | | Autoclaved water + EDTA | | |
|----------|-----------------------------|-----------------------|-------------------|-------------------------|-----------------------|-------------------|
| | Total No. adults | Total No. copepodites | Total No. nauplii | Total No. adults | Total No. copepodites | Total No. nauplii |
| Sept. 22 | 30 | -- | --- | 30 | -- | --- |
| Sept. 29 | 21 | -- | 495 | 25 | -- | 770 |
| Oct. 20 | 49 | 26 | 351 | 21 | 30 | 176 |
| Nov. 17 | 26 | 9 | 250 | 20 | 9 | 93 |
| Dec. 1 | 26 | 18 | 98 | 40 | 25 | 180 |
| Dec. 8 | 41 | 38 | 86 | 60 | 26 | 210 |

Table VIII. Standing stock of a population of Pacific A. clausi affected by EDTA at several times during the period from September 22, 1972 to December 8, 1972.

| Date | Not Autoclaved water - EDTA | | | Autoclaved water - EDTA | | |
|----------|-----------------------------|-----------------------|-------------------|-------------------------|-----------------------|-------------------|
| | Total No. adults | Total No. copepodites | Total No. nauplii | Total No. adults | Total No. copepodites | Total No. nauplii |
| Sept. 22 | 30 | -- | --- | 30 | -- | --- |
| Sept. 29 | 28 | -- | 910 | 25 | -- | 705 |
| Oct. 7 | 15 | -- | 200 | -- | 5 | 93 |
| Oct. 14 | 7 | -- | 51 | -- | 2 | 20 |
| Oct. 20 | -- | -- | 18 | -- | -- | --- |

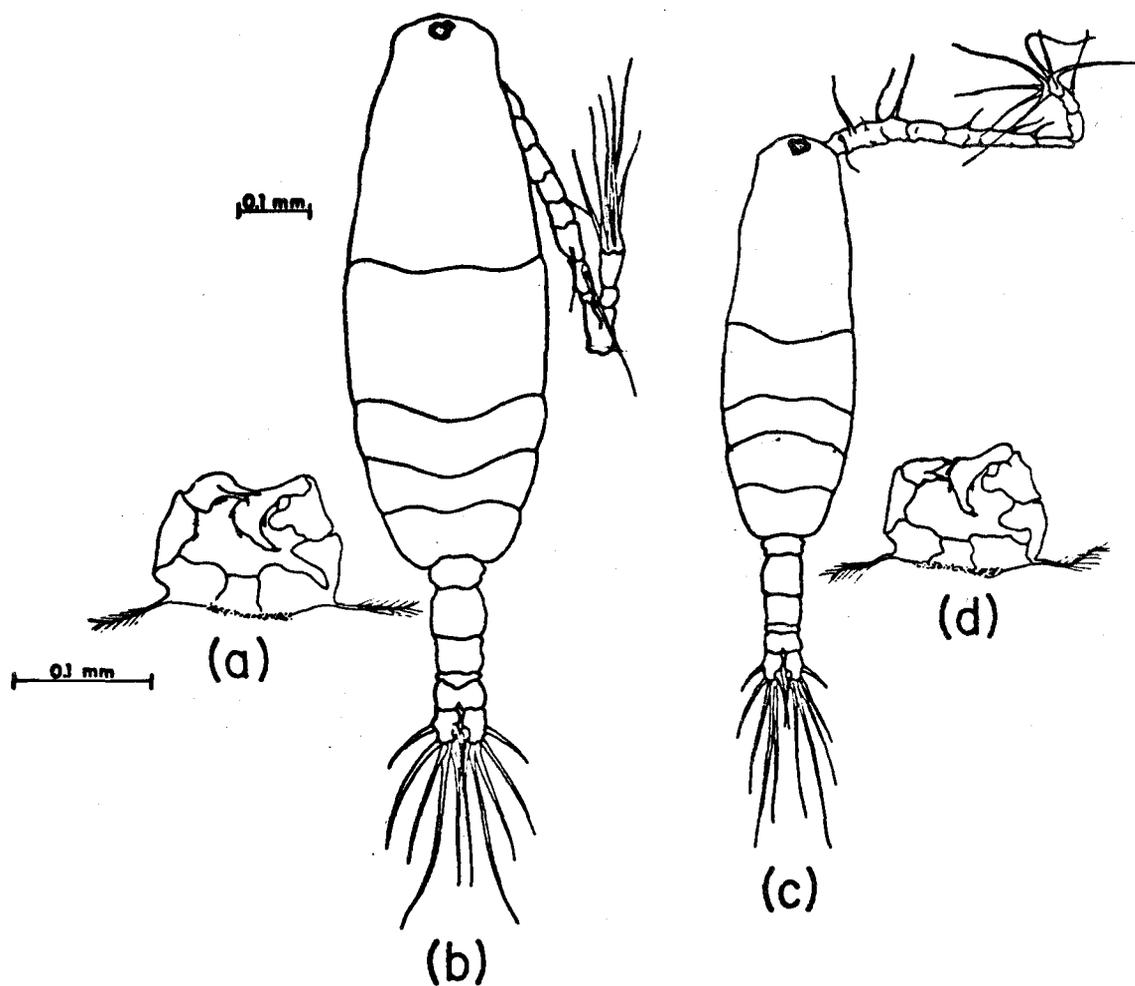


Figure 1. *Acartia clausi*: a) Fifth leg Atlantic male; b) Adult male Atlantic. Dorsal view; c) Adult male Pacific. Dorsal view; d) Fifth leg Pacific male.

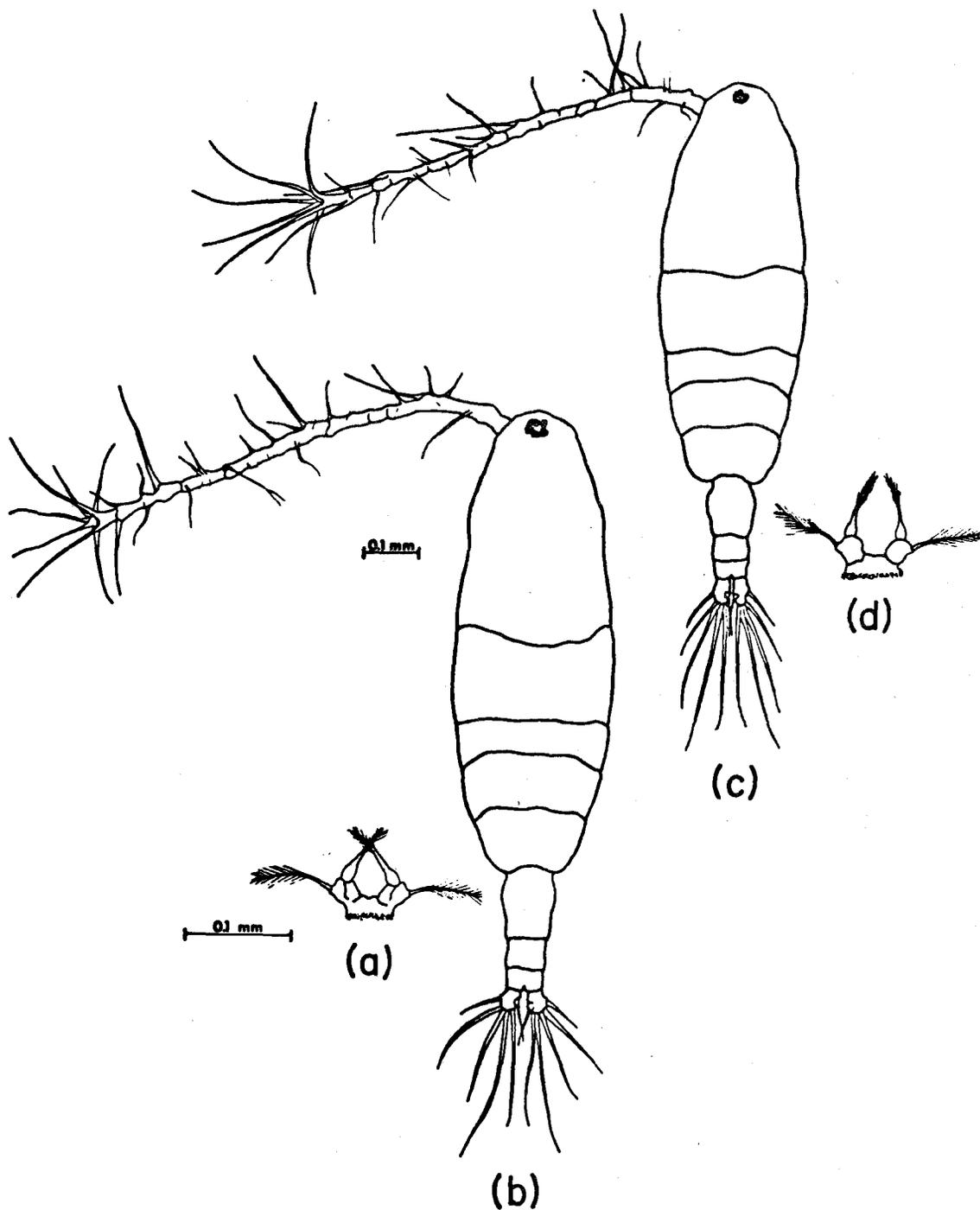


Figure 2. *Acartia clausi*: a) Fifth leg Atlantic female; b) Adult female Atlantic. Dorsal view; c) Adult female Pacific. Dorsal view; d) Fifth leg Pacific female.

larger size difference between the Pacific male and female than between the Atlantic male and female. A difference was found in the shape of the fourth segment of the male urosome. The Atlantic male exhibits a characteristic shape, usually triangular. The Pacific male shows an irregular shape. Fifth legs of the females are very similar in their size and morphology. Fifth legs of the males, on the other hand, are slightly different. Atlantic fifth legs are bigger than Pacific fifth legs, with the penultimate segment of the left leg slightly curved at the middle of the inner margin, showing a hairy termination at that point.

The copepods used for morphological comparison were sampled on July 30, 1973 from cultures started on February 22, 1973 for the Pacific, and June 6, 1973 for the Atlantic.

V. DISCUSSION AND CONCLUSIONS

One objective of this work was to evaluate the morphological and biological species concepts in the assignment of specific rank. A test of these criteria was found in the marine calanoid copepod Acartia clausi. The Atlantic and Pacific populations of this copepod are geographically isolated and have been grouped for many years under the same name, based on morphological similarity (Esterly, 1924; Wilson, 1932). Comparisons made here between cultured copepods of both populations indeed showed remarkably similar individuals, differentiated mostly by size, and by a few more subtle details in the males. Mayr (1963) pointed out that most geographic isolates differ in some morphologic characters. These differences are not necessarily evidence of reproductive isolation, but are frequently correlated with it. If reproductive isolation is proved, then these morphologic features can be used for specific determinations. Whether the observed morphological differences between Atlantic and Pacific A. clausi are constant enough to be used for species recognition is still a matter for a more detailed study.

Based on a detailed review of the literature, Jensen et al. (1969) concluded that temperature "profoundly alters the ultimate size and morphological configuration of certain Copepoda".

It was shown experimentally that under the same environmental conditions, Atlantic and Pacific populations of these morphologically

similar copepods were able to breed and produce fertile offspring. The same was not true when members of the two populations were brought together. Evidently, they have diverged enough to become reproductively isolated. Mayr (1963) discussed the importance of isolation in the formation of species. Among sexually reproducing organisms he recognized between geographical, ecological and reproductive isolates, of which, he concluded, "only the last are species".

Perhaps the occasional presence of fertilized females and hybrid nauplii could suggest the incompleteness of reproductive isolation between these populations. However, Mayr (1963) stated that very few isolating mechanisms are all-or-none devices, and concluded that "species level is reached where the process of speciation has become irreversible, even if some of the isolating mechanisms have not yet reached perfection". Furthermore, Wilson and Brown (1953) stated that even when fertile hybrids are found they do not obscure the existence of two distinct populations, provided hybrids are rare. Mayr (1963) concluded that "if two distinguishable populations of forms with exclusive biparental reproduction occupy the same territory with little or no hybrids, this is ordinarily considered enough to justify the recognition of two species".

Physiological, ecological and behavioral differences have been found in very closely related and similar species. According to Mayr (1963), this indicates "that each species is a separate biological

system with species-specific tolerances to heat, cold, humidity, and other factors of the physical environment, . . . and numerous other biological constants." Indeed, observations on the development of both populations of copepods under the same environmental conditions showed that certain aspects of their biology, ecology and behavior were different. For example, the difficulty to establish a laboratory population of Atlantic A. clausi, the longer developmental time observed for this population, and the higher production of males in the Pacific population. More studies should be made to evaluate these observed differences.

Based primarily on the evidence provided by the interbreeding experiments between individuals of Atlantic and Pacific populations of A. clausi, it is concluded that the populations have diverged enough to become reproductively isolated. Therefore, they should be considered as allopatric sibling species, and no longer as two populations of the same species. The name Acartia muza n. sp. is proposed to designate the Pacific population studied here. However, general acceptance and open publication of this new name to designate Pacific population of Acartia should await further extensive interbreeding and morphologic studies.

In view of these results it can be concluded also (as has been done by several authors) that morphological criteria, per se, are not an absolute tool that systematicists can use to designate species. The

importance of this statement should be understood not only in terms of its taxonomic significance, but in a broader context. Extrapolation of experimental results obtained with a species should be very cautiously applied to other populations included under the same name. For instance, population dynamics data for Atlantic A. clausi (or other species) may not apply to the Pacific form of A. clausi. This is particularly true in the case of the potential construction of a sea level canal connecting the Atlantic and Pacific Oceans (Menzies, 1968; Rubinoff, 1968). Rubinoff (1968) suggested breeding experiments of the kind done in this work as a tool to foresee some of the major biological effects resulting in the mixing of Atlantic and Pacific waters. A detailed discussion of these effects is given by him, but a short paragraph is reproduced here:

"If the isolated populations have completed speciation before they mingle, they may coexist with interbreeding for part or all of their ranges, or competition between the two forms may cause replacement or extinction of one species by the other."

Interbreeding experiments of the kind done in this work should not be difficult or costly to perform. The results obtained could be of help to the taxonomist and those interested in the study of evolution and speciation processes. Decisions affecting ecosystems could be taken with more certainty of the possible outcomes. But more likely, as stated above, costly errors resulting from erroneous assumption of specific and, therefore, ecologic identity could be avoided.

A few examples of ecologically significant differences between the Atlantic and Pacific forms were found in the present culture work. From the summary of culture attempts for Atlantic and Pacific A. clausi found in Table III it can be seen that Pacific copepods were cultured more successfully than Atlantic copepods. Difficulties in establishing an Atlantic culture may be due to several factors. Corkett (1967) suggested that the use of water collected from the same area as the adults influenced their success in the laboratory. Repeated failure to culture Atlantic copepods could be due to the use of water of Pacific ocean origin. However, Pacific copepods were successfully cultured for long periods of time using water collected from different localities indiscriminately. Furthermore, Atlantic A. clausi has lately been cultured successfully for more than two months using exclusively Pacific sea water. Atlantic A. clausi culture had longer development times than those from the Pacific. Battaglia and Lazzaretto (1967) stated that temperature tolerance can be changed genetically and passed on to new generations. If this is the case, then it is possible that the longer development time of Atlantic A. clausi could be the result of genetic adaptation in the parental population to their originally colder environment.

The Pacific cultures produced a greater proportion of males. Jensen et al. (1969) suggested that the ratio of males to females appeared to be higher in species that tolerate warmer waters. Partial

evidence for this suggestion is provided by the fact that indeed the greater number of males (ratio approximately closer to 1) was produced by the Pacific population. This population has been maintained under culture at the relatively warm temperature of 17°C for a longer period of time than the Atlantic population. Therefore, tolerance to warmer waters may have developed for the Pacific copepods. Further observations with the Atlantic copepods could show an increase in the ratio of males to females produced. Heinle (1970) suggested that the ratio of males to females is affected by the density of the population. If that is true, it appears that the effect of density on sex ratio operates with different intensity on Atlantic and Pacific populations, because in this work they were maintained at similar densities. Heinle's hypothesis requires better verification.

A few of the observations from the culture work require some discussion. Early difficulties in the culture of Pacific A. clausi were probably due to the additive effect of several factors, one of which deserves special attention. A strain of Chlorella (NMFS Milford Lab. No. 580) was used as a food item and the possibility of a negative effect on the culture seems likely. Pratt and Fong (1940), Pratt et al. (1944) and Pratt et al. (1945) found out that several species of Chlorella produce and accumulate a substance (designated as chlorellin) that tends to inhibit further multiplication of the cells. They suggested that this substance may well be toxic for certain other organisms. Urry (1965),

in particular, showed that C. stigmatophora Butcher inhibited the feeding and survival of a species of Pseudocalanus.

The possibility of temperature shock was studied in some detail. That several organisms, including copepods, undergo a series of adjustments to sudden temperature changes has been pointed out by Kinne (1957) (cited by Jensen et al. 1969). Because of differences between the temperature of the water where the Atlantic copepods were collected and the final culturing temperature, Atlantic copepods underwent the greatest temperature changes. The highest of these changes (15°C) was recorded for an Atlantic population on March 9, 1973. However, no identifiable effects in activity, health or behavior of the copepods were noticed. The culture simply declined and finally failed. Similar results were apparently observed by Halcrow (1963) with C. finmarchicus subjected to even larger temperature changes. He pointed out the possibility that certain copepods cannot acclimate to temperatures outside the temperature range for the season of capture. His experimental results were in agreement with this, and he found that respiratory acclimation curves for spring and summer populations of C. finmarchicus were different. The culture of Atlantic A. clausi started on March 9 consisted of a winter population collected at water temperatures near 2°C (Table IV). The culturing temperature was probably outside the seasonal temperature range for this winter population, and therefore acclimation to the new temperature may not

have been possible. The success of the Atlantic culture started on June 6, 1973 (summer pop.) could be explained in the opposite terms. Failure observed in the Atlantic culture started on September 8, 1972 (Table V) was shown to be due to the introduction of a new siphon design in the culturing methods. It is possible that, coming from a summer population, these copepods could have been acclimated to the culturing temperature. The physiological basis of possible seasonal differences in temperature acclimation characteristics remains obscure.

The possibility that shipping time could have had an influence in the original survival and further development of copepods is not supported by the evidence. As seen in Table VI, survival of the copepods upon arrival was above 60% independently of shipping time. Further development of these copepods was not hampered as evidenced by the fact that this population (started on September 21, 1972) was successfully cultured for more than three months (Table III).

Neunes and Pongolini (1965) found a reduction in the survival of some copepods reared with autoclaved water. Table VII, however, shows no difference in the survival of copepods reared with autoclaved and non-autoclaved water for a period of more than three months. Presence of EDTA, on the other hand, was evidently a strong factor influencing survival (Table VIII). A detailed study on the effect of this complexing agent is suggested.

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