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Effects of Axenic Culture Conditions on Asexual Reproduction and Metamorphosis in the Symbiotic Scyphozoan *Cassiopea andromeda*

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Abstract

Axexual buds from *Cassiopea andromeda* Forskål 1775 polyps were rendered axenic by a two step antibiotics treatment. Metamorphosis into polyps of bacteria free buds was chemically induced by the peptide carbobenzoxy-Gly-Pro-Gly-Gly-Pro-Ala at 7.3×10^{-5}M. Such polyps were individually raised in test tubes at 23°C and 30°C, and maintained in axenic condition for up to 206 days. A special method was developed to provide polyps with newly hatched, essentially bacteria free *Artemia salina* nauplii. *A. salina* cysts were decapsulated by soaking in sodium hypochloride. Then they were neutralized, rinsed and transferred aseptically to the cultures. Thus, bacteria-free nauplii could be trapped upon hatching from the membranous egg envelope and ingested by the polyps. At 23°C axenic polyps grew to a larger size and started bud production later than the bacterized controls. After shifting the temperature to 30°C budding frequency was constantly increased in axenic polyps but only for about 10 days in the controls. Bacteria-free polyps produced relatively small buds all over the calyx with an up to 10% incidence of malformations such as bifurcated distal end or fused together like Siamese twins or triplets, which never occurred in the controls. Buds from axenic polyps could be chemically induced to metamorphose and provided 2nd and 3rd generation sterile scyphistomae. Normalizing effects of temperature shift from 30°C to 23°C, and of accidental recontamination of axenic polyps are described. The present data constitute the first long-term axenic marine invertebrate predator-prey system.

Keywords: Scyphozoa, *Cassiopea andromeda*, asexual buds, budding, metamorphosis, strobilation, bacteria free culture, axenic predator-prey system

1. Introduction

Cassiopea andromeda Forskål 1775 (Scyphozoa; Rhizostomeae) hosts algal symbionts in both the sessile polyp form and the bottom-dwelling medusa. Contrary to the scyphopolyp which is nutritionally dependent on living animal prey, the shallow water inhabiting medusa is thought to rely predominantly on photosynthates which are transferred from the symbiotic zooxanthellae to the host (Drew, 1972; Hofmann and Kremer, 1981 for review.) As demonstrated earlier in another rhizostome scyphozoan, *Mastigias papua* (Sugiura, 1964), strobilation of medusae by *C. andromeda* polyps occurs only in the presence of endosymbiotic algae (Ludwig, 1969; Hofmann and Kremer, 1981). But it has been shown to occur also in a temperature dependent manner in their absence (Rahat and Adar, 1980). Hofmann et al. (1978), Neumann (1979) and Hofmann and Brand (1987) demonstrated that planula larvae and asexual buds could be induced to enter metamorphosis in the presence of peptides which are thought to be released into the medium through the exocenzymatic activity of the marine bacterium *Vibrio alginolyticus*. These observations led us to ask whether events in the life-cycle of *C. andromeda*, apart from those influenced by algal symbionts, are based on the interaction of this invertebrate with associated marine bacteria. Short-term experiments designed to eliminate bacterial contaminants showed that bacteria-free larvae and buds proved unable to enter metamorphosis (Wolk et al., 1985; Fitt et al., 1987) and required exogenous inducers. However, development was not studied beyond the early polyp stage.

Based on the methods devised by Rahat and Dimentman (1982) for bacteria-free culture of *Hydra* spp. this study aimed at developing a technique for long-term culture of axenic *C. andromeda* polyps in order to study development and asexual reproduction of bacteria- and alga-free individuals, i.e. metamorphosis, polyp morphogenesis, growth, budding and strobilization.

2. Materials and Methods

All experiments reported on in this paper were performed on laboratory reared individuals of *Cassiopea andromeda*. Stock cultures of polyps were maintained at 23°C in natural seawater (from the North Sea) according to methods previously described by Hofmann et al. (1978).

Newly released vegetative buds, less than one day old, were collected, washed three times in ABS_{500} (antibiotics containing seawater, with 500 mg of each penicillin, streptomycin and neomycin dissolved in 1000 ml pasteurized seawater). Then buds were rendered bacteria-free by treating them first for 4 to 6 hr

with ABS_{500} and for 48 hr in ABS_{100} (100 mg of each penicillin and neomycin, and 130 mg streptomycin per 1000 ml). Individual buds were then washed twice in autoclaved seawater and transferred with 1 ml of the same medium into separate test tubes which were covered with Kapsenberg aluminum caps, (see Fig. 1).

Axenic buds were chemically induced to metamorphose into the polyp form by adding an autoclaved solution of the hexapeptide carbobenzoxy-Gly-Pro-Gly-Gly-Pro-Ala in seawater through a sterile, disposable filter unit (pore size 0.22 μm) at a final concentration of 7.3×10^{-5}; some sterilized cotton fibres were added for the buds to settle on. After 2 to 3 days, the young polyps were removed from the peptide solution, rinsed three times, then placed into a fresh, sterile test tube with 10 ml autoclaved seawater. After covering with Kapsenberg caps, the tubes were stored at 23°C. For some experiments the temperature was gradually raised to 30°within 6 days.

To obtain axenic embryos of the brine shrimp *Artemia salina*, cysts were soaked for 30 min and chemically decapsulated in 20% sodium hypochloride, then neutralized through 8 to 10 rinses, each with 10 ml autoclaved seawater and phenol red as a pH-indicator. The sterile, decapsulated embryos, now enclosed only in the inner, membranous layer of the cyst, were stored for up to three weeks at 4°C. They were added to individual, axenic polyps as detailed in the results section (see also Fig. 1).

Sterility tests were performed on samples of culture medium of individual experimental and control buds and polyps, on entire animals and on decapsulated, hatching *A. salina* embryos. Each sample was subdivided and tested in three different nutrient media (described below) at 37°C. The respective material was considered axenic when no bacterial growth occurred in the test media after 7 days. The bacteria-free status could thus only be ascertained *a posteriori.*

Composition of media used for sterility tests:
(a) LM-medium: NaCl 11.7 g; $KH_2PO_4 \times H_2O$ 0.075 g; NH_4Cl 1.0 g; $MgSO_4 \times 7H_2O$ 12.3 g; KCL 0.75 g bacto-peptone (Difco) 5.0 g; yeast extract (MERCK) 5.0 g; glycerol 3.0 ml; Tris-HCl-buffer, pH 7.2 200 ml. (b) NL-medium K_2HPO_4 0.5 g; NH_4Cl 1.0 g; $MgSO_4 \times 7$ H_2O 0.2 g; $FeSO_4 \times 7$ H_2O 0.1 g; $CaCl_2 \times 2$ H_2O 0.01 g; sucrose 10.0 g in 1000 ml distilled water. (c) MB-medium: Marine Broth (Difco) prepared as originally indicated by the manufacturer. Glassware and instruments were heat sterilized for 2 hr at 160°C, media autoclaved at 0.5 bar for 30 min. Disposable dishes and other disposable items were UV-treated before use. All manipulations were carried out under a laminar flow sterile hood while wearing presterilized triflex gloves and a surgical mask.

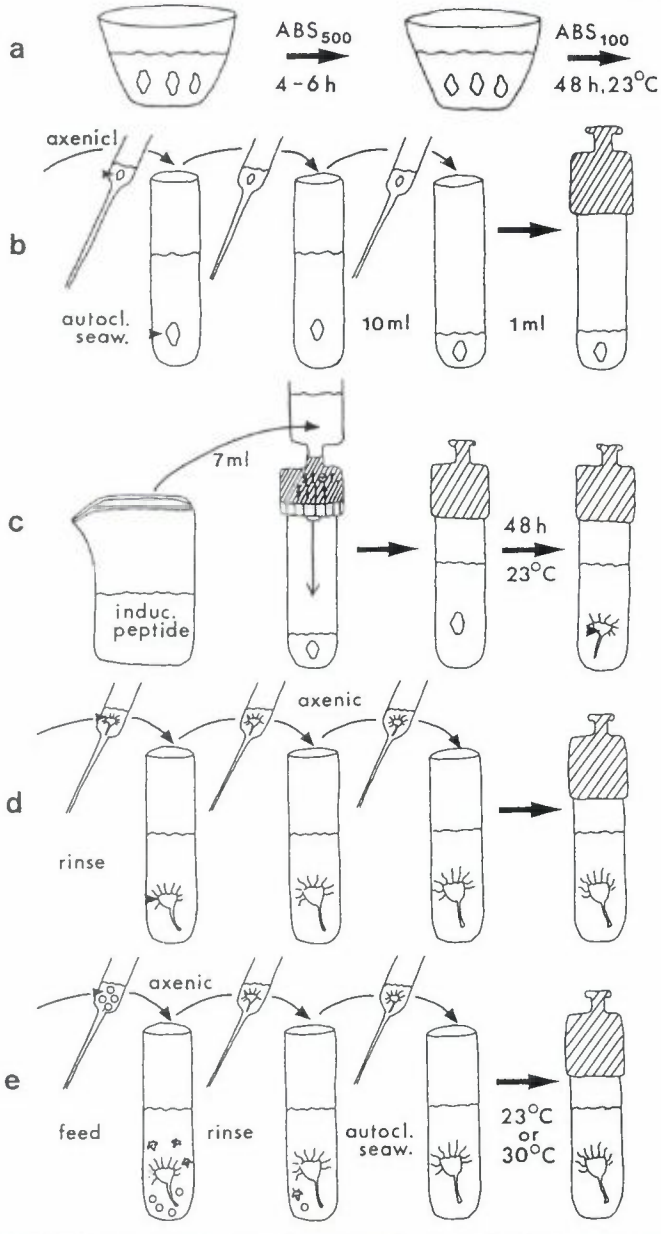

Figure 1. a–e. Schematic representation of axenic culture methods in *Cassiopea andromeda*.
(a) antibiotics treatment of buds
(b) rinsing in and sterile transfer to autoclaved seawater
(c) induction of metamorphosis by Carbobenzoxy-Gly-Pro-Gly-Gly-Pro-Ala under bacteria-free conditions
(d) aseptic transfer from inducer solution to autoclaved seawater
(e) hatching of axenic *Artemia salina* cysts and cleaning of polyps from debris in several rinses with autoclaved seawater

Bud metamorphosis, development of polyps, budding and strobilation was monitored with the aid of a LEITZ-dissecting microscope without removing the individuals from the capped vials. Photomicrographs were taken with an OLYMPUS-IMT2 inverted microscope equipped with an OLYMPUS-OM2 camera.

3. Results and Discussion

Induction of metamorphosis in axenic buds

Following antibiotic treatment in ABS_{500} and ABS_{100} 40 sterile and 40 bacterized buds, each in a separate test tube, were incubated in carbobenzoxy-Gly-Pro-Gly-Gly-Pro-Ala at 7.3×10^{-5}M. Only 70% of the antibiotic treated individuals had performed the typical sequence of metamorphic events after 3 days (see Fig. 2b,c), whereas all controls had metamorphosed within 24 hr. This result is in accordance with Fitt et al. (1987) who recorded a decreased responsiveness to inducing agents following treatment in high concentrations of antibiotics. In an additional series of experiments (data not shown) we observed that buds collected from polyps which were continuously cultured in ABS_{100}, could not be induced to metamorphose, even by a five-fold concentration of the inducer.

Long-term culture on newly metamorphosed polyps

Since scyphopolyps essentially feed on living food organisms the main problem of prolonged axenic culture was to establish a bacteria-free predator-prey system. As a modification of the feeding method applied in the bacteria-free *Hydra* spp. system by Rahat and Dimentman (1982), we introduced about 20 decapsulated bacteria-free *Artemia salina* cysts per axenic polyp. Nauplii hatched from the cysts after 2 or 3 days at 23°C and could be trapped and ingested. Three days after administering the cysts, each polyp was aseptically removed from the test tube and freed from all unhatched material, debris and dead brine shrimp larvae, washed three times in autoclaved seawater, and then placed in a fresh vial. Each polyp was fed and cleaned twice a week for up to 206 days. As controls bacterized polyps were also provided with bacteria-free brine shrimp cysts. Sterility tests were performed at weekly intervals. Four replicates each with 10 buds were rendered axenic and treated with the peptide inducer as shown in Fig. 1. From the total of 40 buds, 13 bacteria-free polyps were obtained. Since the 4 replicates were set up at different times, the time period for which the axenic polyps were maintained at 23°C was not the same for each of the polyps but ranged from 90 to 170 days.

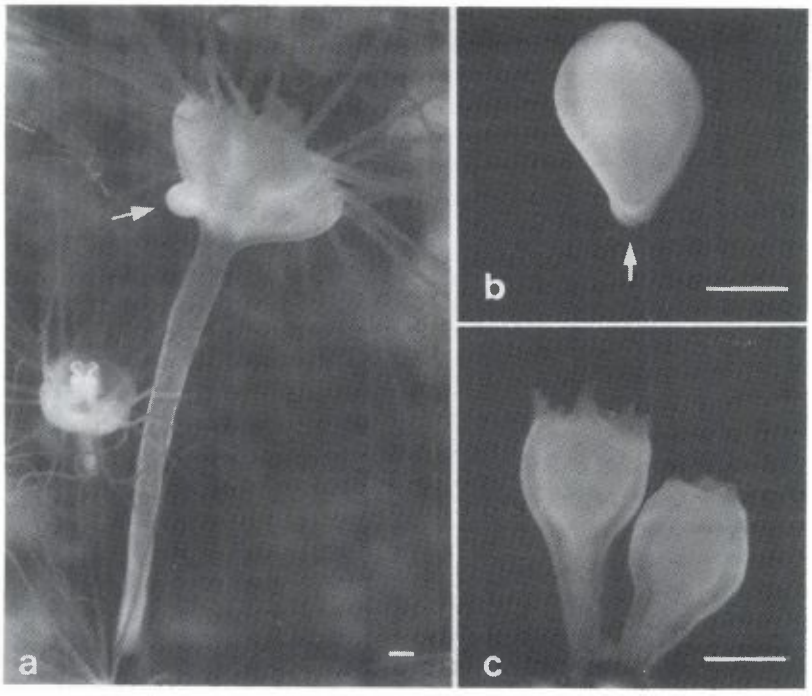

Figure 2. a–c. Budding and bud metamorphosis in *Cassiopea andromeda* under non-sterile conditions.
(a) adult polyp with bud emerging from the lower part of the calyx (arrow)
(b) fully differentiated bud recently released from parent. Arrow points to the presumptive foot region.
(c) two buds settled on empty *Artemia salina* cysts at different stages of metamorphosis. All scale bars = 200 μm.

Growth and asexual reproduction at 23° C

We could not detect any morphological differences between developing bacteria-free and control polyps. Over time, buds were produced in both categories of polyps, however bud formation in axenic individuals was altered in several traits as described below.

Controls

The first bud was released 47 days after metamorphosis of parent polyps. All individuals produced buds and the average budding frequency was 0.7 buds per polyp per day. Budding sites were typically in the lower, perradial areas of the calyx.

Axenic polyps

The first bud was released on day 86 after metamorphosis. Only 8 out of 13 polyps formed buds at an average frequency of 0.8 buds per polyp per day. Budding sites were at the middle and upper regions of the calyx. Before the onset of budding, polyps had grown to almost double the size of control scyphistomae, and the buds of the former were larger since bud size is positively correlated with polyp size.

All buds released from axenic and bacterized control polyps responded to treatment with the inducer peptide carbobenzoxy-Gly-Pro-Gly-Gly-Pro-Ala and metamorphosed normally. Those maintained in autoclaved seawater without the peptide did not metamorphose but kept on swimming, formed only a few tentacle rudiments and a hypostome as described in Hofmann et al. (1978). These buds died after 3 to 5 weeks. Minor aberrations of bud shape occurred in less than 10% of axenic and non-sterile buds.

Five axenic polyps metamorphosed from axenic buds, which had not been treated with antibiotics, were maintained as a second generation for up to 70 days. All of them developed buds. From such buds a third generation of axenic polyps were raised.

Budding and strobilation at 30° C

Monodiscous strobilation of medusae occurs only rarely at 23°C in our mass-cultures of *Cassiopea andromeda* polyps. It was not observed at this temperature in our experiments reported in this paper. However, it is known from several investigations (Hofmann et al., 1978; Rahat and Adar, 1980; Hofmann and Kremer, 1981) that strobilization is enhanced at higher temperatures whereas budding is concomitantly quenched.

To test whether the ability of polyps to form medusae and to release buds at higher temperature, 13 axenic and 5 control polyps kept for 90 to 178 days at 23°C, were transferred to an ambient temperature of 30°C. This temperature is within the normal range encountered by *C. andromeda* in its natural habitats. Within 5 weeks after raising the temperature, none of the 13 axenic polyps and only 1 of the 5 controls formed a young medusa. At the end of the experiments all polyps were screened under an epifluorescence microscope for chlorophyll *a* of algal symbionts. Only the scyphistoma from the control series which had strobilated was found to contain zooxanthellae. The transfer to 30°drastically affected bud formation in several ways. In addition to a sudden increase of the budding frequency to 4.6 buds per polyp per day, including bud formation in those which had not produced buds before, axenic polyps lost the distinct

shape of the calyx (Fig. 2a) and their form became irregular. Budding was not restricted to the 4 perradial areas close to the stalk but occurred all over the calyx. Buds were smaller (< 0.5 mm) than those of the controls (0.5 to 0.7 mm). Abnormal axenic buds with either bifurcated distal end (i.e. the presumptive basal portion of the polyp), or fused together like Siamese twins or triplets, were common (5 to 10%). The budding frequency at 30°C remained high throughout the observation period of 5 weeks at an average of 4.6 buds per polyp per day compared to 0.8 at 23°C.

Bacterized controls also increased bud production at 30°C but only for 10 days after which they turned to the low level of budding seen in animals maintained at 23°C. A similar observation was reported by Hofmann et al. (1978). In this particular case (*loc. cit.*, Fig. 2) however, decline of budding was followed by the onset of strobilation. In the present experiments only one of the controls strobilated. Buds with peculiar abnormalities, as seen in axenic parents, never occurred. The average budding frequency was 0.9 buds per polyp per day.

All buds which were released at 30°C metamorphosed when induced with the bioactive peptide. Normal buds developed into typical polyps and abnormal buds gave rise to different types of malformed scyphistomae (e.g. with duplicated stalk or with duplicated and even triplicated calyx).

Five normally shaped second generation axenic scyphistomae were selected and maintained at 30°C. The first individual started budding at this temperature 33 days after metamorphosis. Buds from these polyps were induced to metamorphose and were cultured as third generation axenic polyps.

Bud formation after a return from 30°C to 23°C

After 5 weeks at 30°C, 8 axenic polyps were transferred back to 23°C for another 38 to 60 days. Budding activity immediately decreased at the lower temperature; the average frequency went from 4.6 to 0.3 buds per polyp per day. No bifurcated or twinned buds appeared any further and the polyps gradually resumed their typical shape. Figure 3 shows data from one replicate of axenic and control polyps which were returned from 30°C to 23°C for 38 days.

Effects of recontamination of axenic polyps

The successful establishment of long-term axenic culture of *Cassiopea andromeda* allows to (re)introduce known species of marine bacteria, subfractions or metabolites or bacteria, and of various bioactive compounds. This will clarify the specific role of the bacterial epi- and endoflora in development and asexual reproduction of a marine invertebrate.

In the present work preliminary information is available only from accidental recontamination with unknown bacteria of three polyps. Two polyps became bacterized at day 180, i.e. 8 days before the end of the observation period, and were not investigated further. However one polyp became non-sterile shortly after being transferred from 23°C to 30°C. As shown in Fig. 3, recontamination was related to a reduced budding rate of 0.65 buds per polyp per day compared

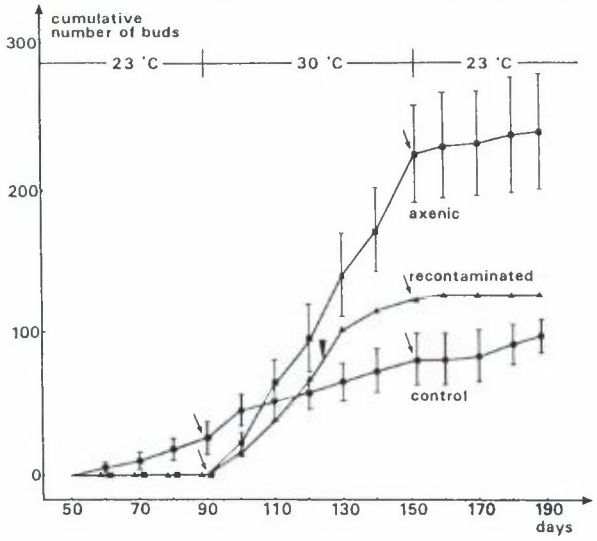

Figure 3. Budding activity in *Cassiopea andromeda* polyps at different temperatures observed under axenic, recontaminated and non-sterile conditions. Axenic (n=3) and recontaminated (n=1) polyps are from the same replicate of 4 individuals. Graphs represent mean values of axenic polyps and of bacterized controls (n=3), and individual data of the recontaminated polyp. For details see text. Arrows: date of temperature shift. Arrowhead: detection of bacterial contamination by sterility test. Bars: ±S.D.

to a mean rate of 2.62 in the axenic polyps between day 130 and day 152. Thus the budding rate in the recontaminated individual was close to that of the controls which was 0.47.

Comparison with other axenic systems

Attempts to obtain axenic larve with or without the use of antibiotics have been made in *Cassiopea andromeda* (Wolk et al., 1985), in *Ostrea edulis* (Millar and Scott, 1967) and *Strongylocentrotus* (Manahan et al., 1982). *C. andromeda* is the first marine invertebrate which has been successfully introduced in a long-term axenic culture system. *Hydra* spp. appear to be the first limnic invertebrate cultured axenically (Rahat and Dimentman, 1982). Whereas both

symbiotic and aposymbiotic *Hydra viridis* readily formed buds in bacteria-free culture, axenic polyps from two non-symbiotic strains of *Hydra viridis* were unable to produce buds during a period of 4 weeks (Rahat and Dimentman, 1982). But the latter species resumed normal budding 1 to 3 days after introducing bacterized *Artemia salina* nauplii or after inoculation with bacteria isolated from stock-cultures. Rahat and Dimentman (1987) concluded from these results the absence of an intrinsic budding factor. They state that the factor can be reintroduced by non-sterile food organisms. In *H. viridis* however, a budding factor was considered to be endogenous and constitutive by these authors.

Axenic polyps of *Cassiopea andromeda* tend to react more like *Hydra viridis*, but nevertheless show how two remarkable differences. In young polyps at 23°C, growth dominates over bud formation for many weeks. After temperature shift to 30°C the entire system which controls budding appears to be affected. This suggests that the normal bacterial complement of polyps does not interact with the host in a simple all-or-nothing manner. However, possible pathways of interaction still need to be elucidated. In our study aposymbiotic, axenic scyphopolyps were incapable of strobilating medusae. This result is compatible with observations by Ludwig (1969) and by Hofmann and Kremer (1981) who found that aposymbiotic *Cassiopea andromeda* polyps did not strobilate. In contrast, Rahat and Adar (1980) observed medusa formation by aposymbiotic polyps within 28 to 40 days after transfer from 18 ± 2°C to 20°–26°C and already within 22 to 30 days when transferred to 28°–30°C.

Taken into consideration that in axenic polyps at 23°C, budding occurred after 86 days at the earliest and that at 30°C polyps of the 2nd axenic generation started budding only after 34 days, the available time period of 35 days might have been too short to allow strobilation in axenic, aposymbiotic individuals. Since our present data are based on a small sample size, we cannot rule out completely the possibility that axenic, aposymbiotic polyps might have the potential to strobilate medusae.

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REFERENCES

Drew, E.A. 1972. The biology and physiology of alga-invertebrate symbiosis. I. Carbon fixation in *Cassiopea* sp. at Aldabra atoll. *J. Exp. Mar. Biol. Ecol.* **9:** 65–69.

Fitt, W.K., Hofmann, D.K., Wolk, M., and Rahat, M. 1987. Requirement of exogenous inducers for metamorphosis of axenic larvae and buds of *Cassiopea andromeda* (Cnidaria: Scyphozoa). *Mar. Biol.* **94:** 415–422.

Hofmann, D.K. and Brand, U. 1987. Induction of metamorphosis in the symbiotic Scyphozoan *Cassiopea andromeda*: Role of marine bacteria and of biochemicals. *Symbiosis* **4:** 99–116.

Hofmann, D.K. and Kremer, B.P. 1981. Carbon metabolism and strobilation in *Cassiopea andromeda* (Cnidaria, Scyphozoa): Significance of endosymbiotic dinoflagellates. *Mar. Biol.* **65:** 25–33.

Hofmann, D.K., Neumann, R. and Henne, K. 1978. Strobilation, budding and initiation of Scyphostoma morphogenesis in the Rhizostome *Cassiopea andromeda* (Cnidaria: Scyphozoa). *Mar. Biol.* **47:** 161–176.

Ludwig, F.-D. 1969. Die Zooxanthellen bei *Cassiopea andromeda* Eschscholtz 1829 (Polyp-Stadium) und ihre Bedeutung für die Strobilation. *Zool. Jb. (Abt. Anat. Ontog. Tiere)* **86:** 238–277.

Manahan, D.T. and Davis, J.P. 1983a. Bacteria-free sea urchin larvae: Selective uptake of neutral amino acids from seawater. *Science* **220:** 204–206.

Millar, R.H. and Scott, J.M. 1967. Bacteria-free culture of *Oyster* larvae. *Nature* **216:** 1139–1140.

Neumann, R. 1979. Bacterial induction of settlement and metamorphosis in the planula larvae of *Cassiopea andromeda* (Cnidaria: Scyphozoa, Rhizostomeae). *Mar. Ecol. Prog. Ser.* **1:** 21–28.

Rahat, M. and Adar, O. 1980. Effect of symbiotic zooxanthellae and temperature on budding and strobilation in *Cassiopea andromeda* (Eschscholtz). *Biol. Bull.* **159:** 394–401.

Rahat, M. and Dimentman, Ch. 1982. Cultivation of bacteria-free *Hydra viridis*: Missing budding factor in nonsymbiotic Hydra. *Science* **216:** 67–68.

Sugiura, Y. 1964. On the life-history of rhizostome medusae. II. Indispensability of zooxanthellae for strobilation in *Mastigias papua*. *Embryologia* **8:** 223–233.

Wolk, M., Rahat, M., Fitt, W.K., and Hofmann, D.K. 1985. Cholera toxin and thyrotropine can replace natural inducers required for the metamorphosis of larvae and buds of the scyphozoan *Cassiopea andromeda*. *Roux's Arch. Dev. Biol.* **194:** 487–490.