STUDIES ON THE DAILY RHYTHMIC ACTIVITY OF THE SEA-PEN, *CAVERNULARIA OBESA* VALENCIENNES. XVIII. ONTOGENIC DEVELOPMENT OF THE DAILY RHYTHMIC ACTIVITY[1)]

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With 8 Text-figures

This paper is dedicated to
Prof. Jürgen ASCHOFF for celebrating of his 60th birthday.

A great deal of works have hitherto been published about the daily rhythmic activity of animals and the circadian rhythm research seems to have recently become one of the most important themes in the biological science. Some of the important works are introduced in such books as CLOUDSLEY-THOMPSON (1961), BÜNNING (1963), ASCHOFF (1965), SOLLBERGER (1965), MAYERBACH (1967) and CONROY and MILLS (1970).

However, relatively little attention has hitherto been paid to the ontogenic development of the daily rhythmic activity, irrespective of importance of the problem. When we attempt to clarify the origin of the circadian rhythm, we have to study the ontogenic development of it. As for the literature concerning this side of work are briefly summarized by SOLLBERGER (1965), RENSING (1965) and PETREN and SOLLBERGER (1967). Most of these works described only the ontogenic appearance of daily rhythmic phenomena through a life of an individual. For example, MORI (1949) studied the changes in the daily rhythmic phenomena through a life of an individual of *Drosophila melanogaster* MEIGEN as for egg laying, hatching, molting, pupating and emergence, MATSUTANI and MORI (1950) described the ontogenic development of daily rhythmic activity of a land snail, *Fruticicola sieboldiana* (PFEIFFER), or ONDO (1954) made research with a pill-bug, *Tylos granulatus* MIERS, on the change of the daily activity through the development.

On the other hand, only very few investigations were attempted to analyse the process of establishment of the endogenous nature. PETREN and SOLLBERGER (1967) summarized their works on the development of internal physiological rhythm—liver glycogen rhythm, and discussed about this matter. But they did scarcely touch with the activity rhythm of whole animal.

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Besides, there are some works concerned hereditary nature of circadian rhythms. These were referred and discussed by BÜNNING (1963) formerly and by MORI, SUZUKI and YANAGISHIMA (1964) later. Although those hereditary phenomena are related to the ontogenic development of spontaneous circadian rhythm, they are not treated in this paper because of lack of data with the sea-pen.

The present paper deals with the ontogenic development of the endogenous daily activity rhythm in the sea-pen, *Cavernularia obesa* VALENCIENNES. The senior author has long been studying the daily rhythmic activity in adult of this coelenterate and the results obtained up to 1960 were summarized and announced at the Cold Spring Harbor Symposium.

Early in October of 1942 the senior author noticed in an aquarium of the Seto Marine Biological Laboratory that some colonies did not contract and continued expansion above the sand after sunrise, when they used to contract in the normal case (MORI, 1944). At about 2 or 3 hours after sunrise they suddenly began to spawn eggs or to ejaculate sperms, which lasted about half or an hour. Finishing the sexual behavior, they contracted quickly beneath the sand. The breeding period extended about a month.

At the present experiment we get fertilized eggs from those colonies reared in an aquarium. The method for culturing the larvae were searched in October of 1965 and the preliminary observation on the ontogenic development of the daily rhythm was made at that time. The main experiments were performed in October of 1966. All observations and experiments were done at the Seto Marine Biological Laboratory of Kyoto University.

The authors wish to express their sincere thanks to the staff of the Seto Marine Biological Laboratory for their kind aids in offering facilities and in many ways.

General Descripton of Early Developmental Stages

Several tens or hundreds of fertilized eggs were put into a glass finger bowl (diameter 10 cm, height 5 cm) with sea water and placed in running water in order to prevent an extreme fluctuation of water temperature. Water in the finger bowl was renewed once a day.

The process of development was observed under a binocular microscope at three hour intervals. The general feature was as follows (refer to Figs. 1 and 2).

xth day morning: spawning eggs and ejaculating sperms—fertilization (Fig. 1–*a*).

xth day afternoon: 16 cells stage—morula stage (Fig. 1–*b*,*c*).

(x+**1**)th day: blastula—planura larva; first, stay on the bottom of the vessel, then gradually float up (Fig. 1–*d*).

(x+**2**)th day: "rachis-like and peduncle-like" parts are gradually differentiated; float and swim; peristalsis is not observable.

(x+**3**)th day: some individuals swim about and others aggregate on the vessel wall near water surface; no one is on the bottom; peristalsis appears (Fig. 1–*e*).

(x+**4**)th day: swimming activity gradually weakens; some sink on the bottom, but not adhere to it.

(x+**5**)th day: original upheaval of tentacles becomes distinct and peristalsis can clearly be observed in well developed individuals; some animals swim slowly and others move on the bottom; adhering tendency to the bottom appears.

(x+**6**)th day: small warts (rudiments of ramuli) appear on the tentacles of well developed individuals; individual differences in development become remarkable.

(x+**7**,**8**)th day: *daily rhythmic contraction and expansion of tentacles can clearly be observed;* other parts of polyp also seem to show a slight tendency of daily rhythmic contraction and expansion, but not remarkably; new rudimentary autozoids appear.

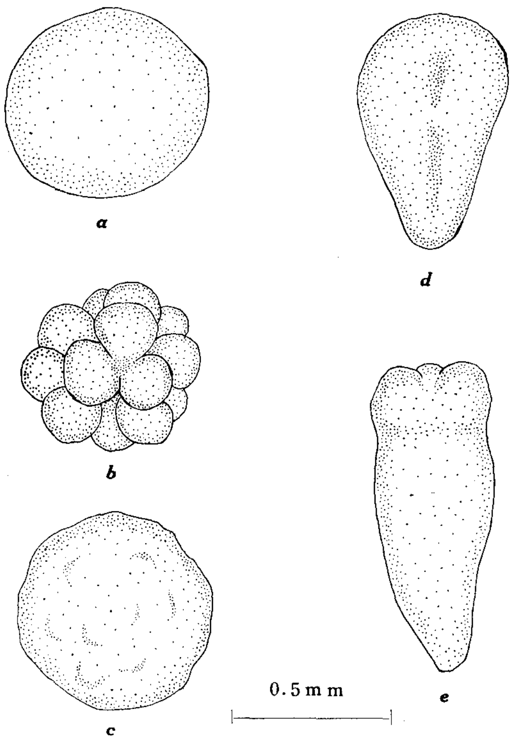

Fig. 1. *a*: Fertilized egg (xth day morning).
b: 16 cells stage (xth day noon).
c: Morula stage (xth day afternoon).
d: Planura larva in (x+1)th day.
e: Larva in (x+3)th day.

(x+**10**)th day: tentacles elongate, ramification of which becomes clear; two new autozoids distinctly protrude, but not yet grow up to regular polyps (Fig. 2–*a*,*b*,*c*).

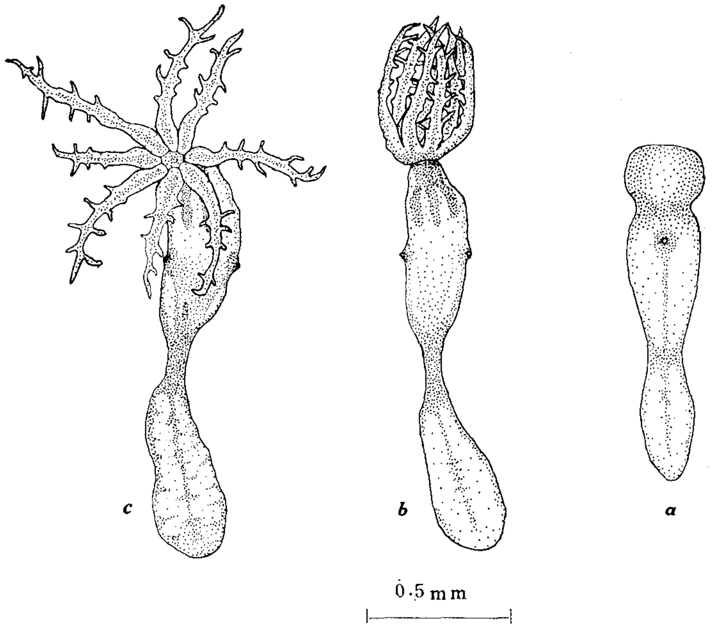

Fig. 2. Polyp stage larva.
a: Contracted resting state in the daytime (form 1)
b: Intermediate state (form 3)
c: Expanded active state at night (form 5)

We could rear the young animals more than a month in the bowl, but unfortunately the differentiation did essentially not proceed beyond the condition mentioned above, and they even gradually weakened. The scantiness of suitable food was probably be causative, as we could not find out any appropriate food for them.

Features of the Daily Rhythmic Activity at the Early Developmental Stages

As described in the previous paragraph the daily rhythmic activity comes in sight just when the tentacles differentiate, about 7 or 8 days after fertilization. Furthermore, this activity develops first in the tentacles and rapidly becomes clear as they develop; the daily rhythmic contraction and expansion of the whole body, which are the conspicuous mode of activity of the adult animals, can also be observed but not so distinctly in the young animal of polyp stage. The peristalsis appears relatively early in the development (3 days after fertilization), but the daily rhythmic change of its direction,

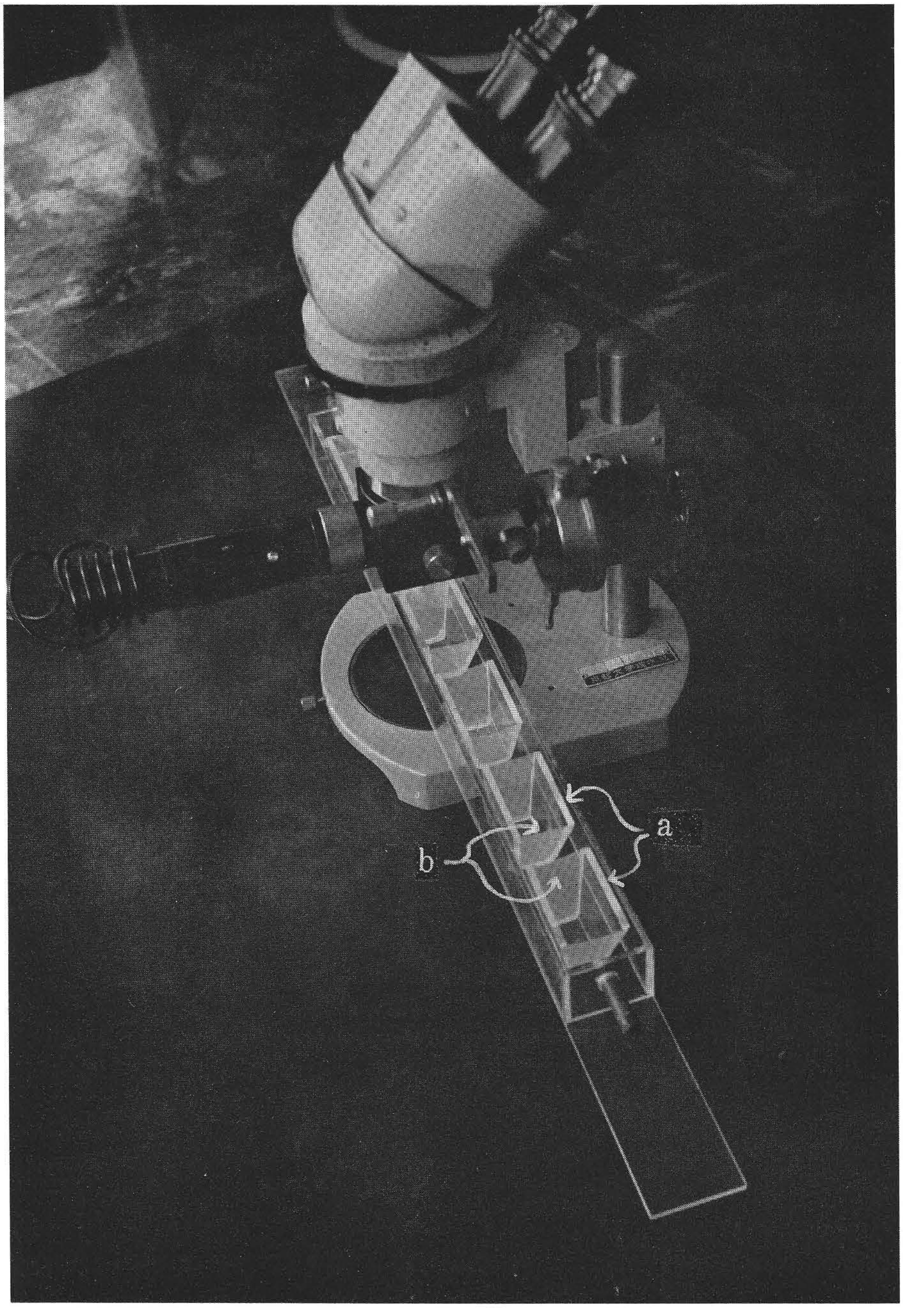

Fig. 3. Several small plastic vessels placed in a row in the plastic trough.
a: plastic wall. b: Müller gauze screen.

which is very distinct in the adult animals (MORI, 1943), remains obscure through the polyp stage.[4)]

In order to observe more clearly and exactly the features of the daily rhythmic activity, we prepared a culture vessel as shown in Fig. 3. It was made of a plastic trough (length 42 cm, height 3.5 cm, width 4 cm) and 5 or 6 small plastic vessels were placed in it. The height of this small experimental vessel was 3 cm, and the extent was a little larger at the top (3×5 cm) than on the bottom (2×3 cm) to make the use of a binocular microscope easier. The two lesser sides of the small vessel had no plastic walls, but were covered by 0.33 mm mesh Müller gauze to keep the flowing of sea water in the trough through the small vessels.

Ten to twenty individuals of the same developmental stage were transferred in each experimental vessel from the glass bowls in which stock individuals were being cultured as mentioned above. The developmental stages of the animals when put in the experimental vessels ranged from just after the fertilization to about two weeks after the fertilization. The activity states were observed by a binocular microscope at one to three hour intervals through a few days keeping the animals continuously in the same experimental vessels. The spot light used at night observation was controlled to be as weak as possible in intensity and as short as possible in time, as far as the exact observation was ensured.

Fig. 2–*a* and *c* show a typical rest condition in the daytime and a typical active condition at night of the tentaculated larva, respectively. It is clear that the tentacles contract in the daytime and well expand at night.

In order to show the degree of activity we used an activity index. We distinguished five states of the body form in daily activity, the states 5 and 1 are both shown in Fig. 2, the former (the extremely active, expanded state) as *c* and the latter (the extremely rest, contracted state) as *a* there. Between these two extreme states three intermediate states were defined, they are, states 4, 3 (Fig. 2–*b*) and 2, thus we used these five states as the activity indices.

Experimental Procedures and Results

The young animals, about 6 days, 9 days, 15 days and 20 days after the fertilization respectively, were divided into two groups, and one of which was maintained under normal day-night condition, while the other was transferred into a dark room and maintained under constant darkness.

1. Normal Rhythmic Activity

After the animals had developed tentacles they showed the daily rhythmic activity. Fig. 4 shows typical features of normal day-night activity of three individuals. The

4) Adults show upward and downward peristalsis when they are expanding and contracting respectively.

rhythm is not so remarkable as in the case of adult colony, yet at least, the day-contraction and the night-expansion of tentacles are clearly seen.

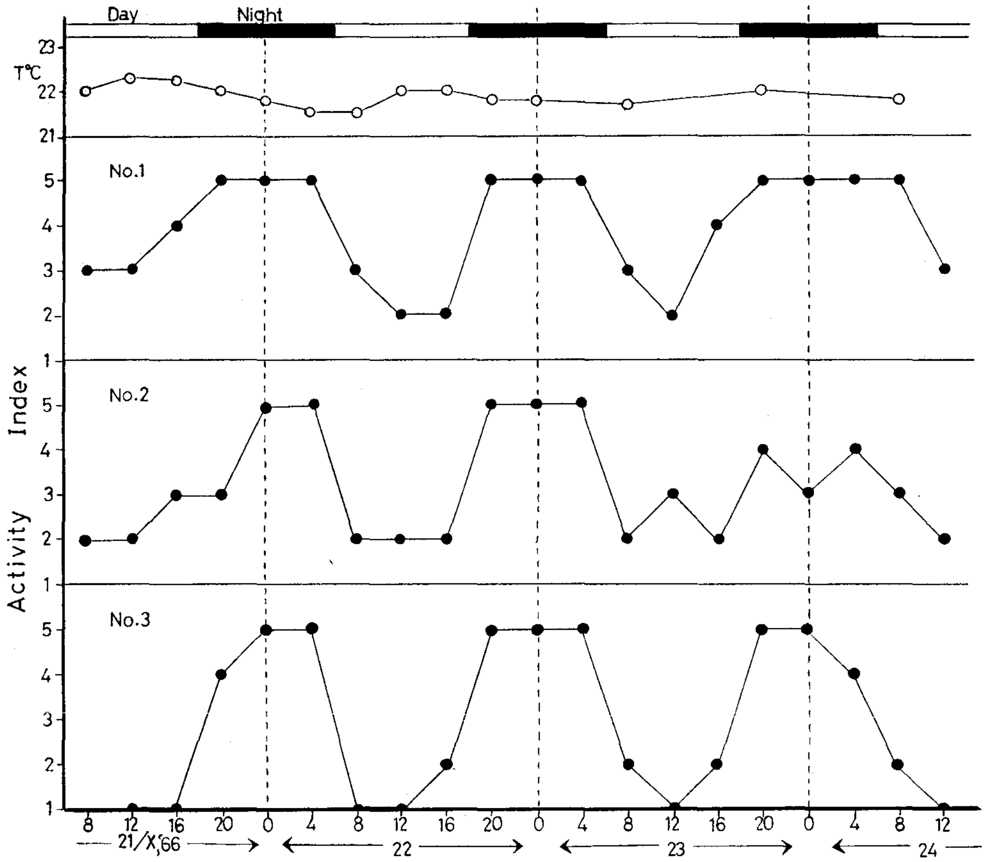

Fig. 4. Normal daily rhythmic activity of three individuals which were born on the 14th, October. Ordinate: Activity indices exhibited by tentacles. Upper column shows environmental light condition (in this case natural day-night time schedule).

2. *Activity under Constant Darkness*

It is very important to see the mode of activity under constant darkness, because we can see the endogenous nature, at least partly, by this experiment.

Fig. 5 shows the activities under constant darkness of two individuals, which were born on the 13th October, 1966, and reared under natural day-night condition until the 19th (6 days after fertilization) when they were put in the dark room and kept under constant darkness. The tentacles were developed at 8:00 on the 20th in dark and the data of activity index were registered after 20:00 on the same day. The fluctuation of water temperature was maintained within 1.5 °C through the experiment.

As is seen in this figure, no circadian rhythm is observable.

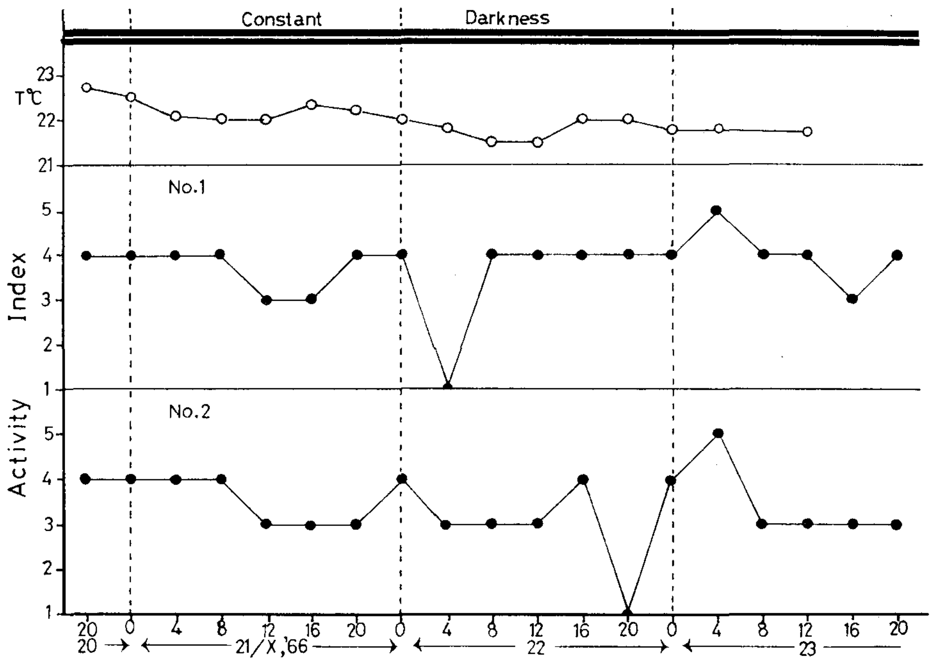

Fig. 5. Activity under constant darkness of two individuals which were born on the 13th, October. They were placed in constant darkness at 10:00 on the 19th. Tentacles developed at 8:00 on the 20th.

Fig. 6 shows the activities of three individuals which were born on the 19th October, 1966, and reared under natural day-night condition until 10:00 on the 28th (9 days after fertilization). The tentacles developed on the 26th or 27th. The circadian rhythm seems to last at least for one day after they were brought into constant darkness. The feature is different from that in the former case (the tentacles developed in dark), that is, the endogenous nature is, though not strongly, developed in this case.

Fig. 7 shows the activities of two individuals which were born on the 19th October, 1966, and reared under natural day-night condition until 13:00 on the 3rd November (15 days after fertilization) when they were put in the dark room. The circadian rhythm of No. 2 individual persists at least 2 or 3 days, this shows a stronger development of endogenous character than in the former cases. As for the mode of activity of No. 1 individual, persistence of the rhythm for one day in dark is clear but afterwards the rhythm becomes obscure.

Fig. 8 shows the activities of three individuals that were born on the 14th October, 1966, and reared under natural day-night condition until 10:00 on the 3rd November (20 days after fertilization) when they were put in constant darkness. Generally

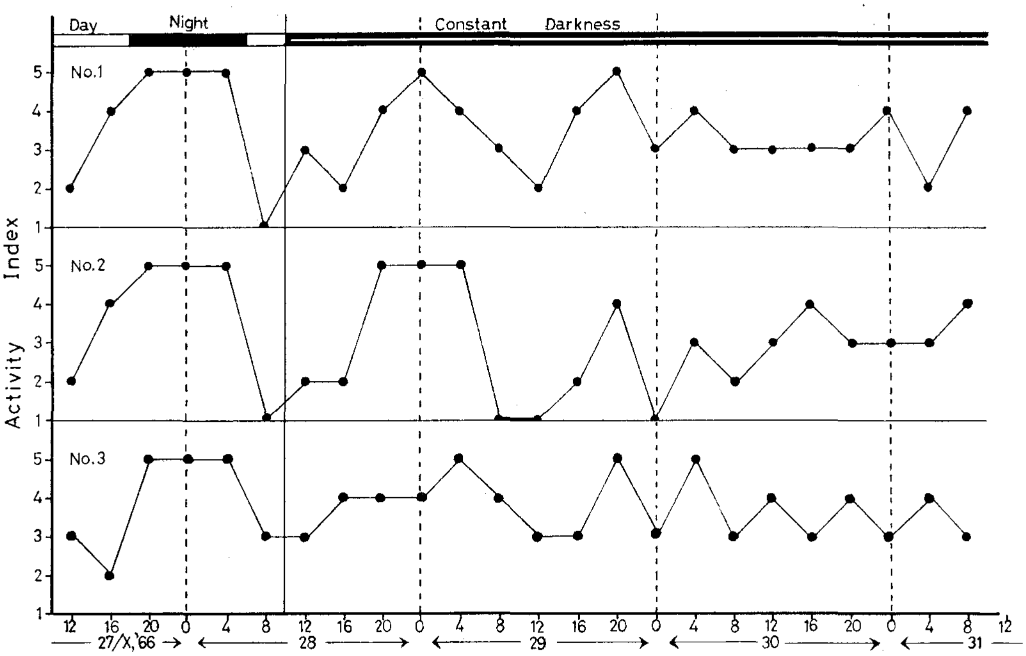

Fig. 6. Activities under constant darkness of three individuals which were born on the 19th, October. They were placed in constant darkness at 10:00 on the 28th.

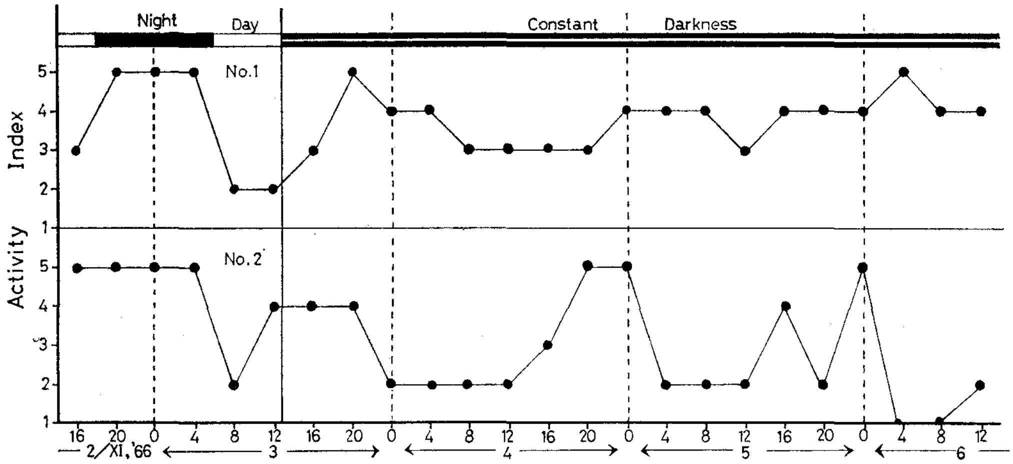

Fig. 7. Activities under constant darkness of two individuals which were born on the 19th October and were placed in constant darkness at 13:00 on the 3rd November.

speaking, it was difficult to maintain the animal in a healthy condition more than 20 days after fertilization, so that it will be safe to consider this experiment only for supplement. In any way, the individual No. 2 seems to show the persistence of circadian rhythm of at least 2 cycles; the rhythmic nature of No. 3 individual is maintained for 3 days, though the cycle is remarkably prolonged; the rhythm of No. 1 individual is maintained only for one day in dark and then it is disturbed.

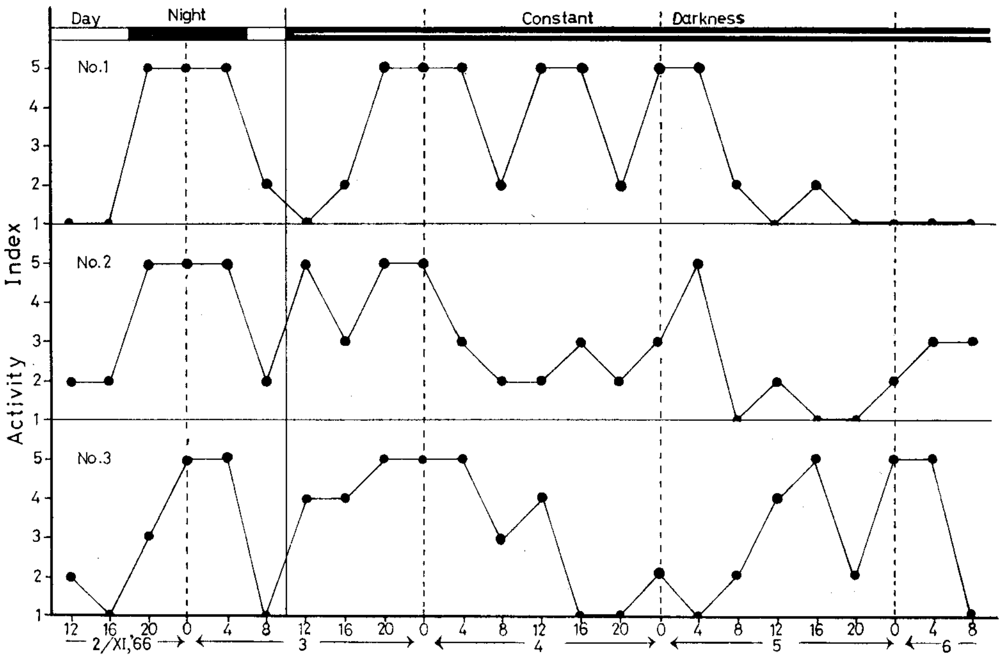

Fig. 8. Activities under constant darkness of three individuals which were born on the 14th October and were placed in constant darkness at 10:00 on the 3rd November.

Considerations

It is frequently observed that the type of daily activity is arhythmic in younger stages of development, or if any, not so remarkably developed as in the adult stage, or has a pattern different from that of the adult (RENSING, 1964). For example, the daily locomotor activity of a young land snail, *Fruticicola* (*Acusta*) *sieboldiana* (PFEIFFER), is arhythmic, but gradually becomes nocturnal as it grows (MATSUTANI and MORI, 1950). The circadian rhythm of *Tylos granulatus* MIERS is observable in individuals less than 5 mm in body length but it is not so conspicuous as in adults (ONDO, 1954). In some case a rhythm of mother affects simply a rhythm of young, that has no overt daily rhythm, and forces the young to behave as if it had a daily rhythm. For example, adult females of a fruitfly, *Drosophila melanogaster* MEIGEN, lay eggs in the evening and most eggs hatch in the next evening and become the 1st instar larvae. The ecdysis of 40% of the 1st instar larvae occurs in the forenoon on the 3rd day after hatching, but the ecdysis of the rest individuals takes place extending 2 days. The ecdysis of 30% of the 2nd instar larvae occurs in the forenoon on the 4th day after hatching, but the ecdysis of the rest individuals takes place in subsequent 4 days. Thus the daily rhythmic pattern becomes more and more obscure as the larval development proceeds until the larvae grow up to pupae which show a clear daily emergence rhythm because of differentiating the definite organ (eye) for reacting to light change (MORI, 1949).

In the present case, the young larvae of *Cavernularia obesa* are arhythmic as in many cases of other animals, but exhibit rather suddenly the daily rhythmic behavior when they grow up to the stage of differentiating tentacles.

SOLLBERGER (1965) considered on possibilities for the formation of rhythms through developmental stages and enumerated several cases. The case of the sea-pen seems to be related to the category 5 in his citation, which points out that "the rhythm cannot appear until neural and endocrine apparatus has matured". We should like to include sensory apparatus in addition to neural and endocrine apparatus in SOLLBERGER's sentence. Really MORI observed frequently in the adult sea-pen that the tentacles of autozoid reacted more sensitively to light change than any other parts of the body.

In any way, the fundamental importance is in formation of organization of living systems, especially nervous and sensory systems, for the development of daily rhythmic activity. In accord with the accomplishment of organizations the exhibition of daily rhythm becomes clear.

In this connection, no description has hitherto been given, so far as we are aware, on the development of endogenous nature of the circadian rhythm. Our research shows that the endogenous character is gradually established as the development proceeds, i.e., the autonomy of the circadian rhythm is more and more firmly established as the development proceeds.

The role of environmental rhythm on the formation of daily activity rhythm may also be important and the synchronization of the organismic rhythm with the environmental rhythm will generally be an essential condition for the normal development of the daily rhythmic activity. In the present observations, the individuals of the sea-pen, which placed in constant darkness on the 6th day after fertilization, that is, before the formation of tentacles, did not show any circadian rhythm of activity. This seems to indicate that for the appearance of the normal rhythmic activity through development, it is necessary being triggered by the environmental rhythm when appropriate receptors, adjustors or effectors are formed.

Although it is very important to see how hereditary natures are concerning the development of daily rhythmic activities, we cannot touch the problem at present, because we could not rear a newly born sea-pen in healthy condition more than 20 days. ASCHOFF (1955) kept mice for several generations in constant condition and BROWMAN

(1952) also kept rats for 25 generations in constant darkness, and both said that these animals still maintained their circadian rhythms. In order to see this kind of phenomena in the sea-pen and to detect the relations among the development of the animal, the individual development of the daily rhythmic activity and the hereditary nature, we have to find out an appropriate technic of rearing the animal for long time up to the adult form. This is the most important further problem.

Summary

1. The sea-pen, *Cavernularia obesa* VALENCIENNES, spawns in October and the eggs are fertilized in water and start to develop instantly.

2. In earlier stages of development they do not exhibit daily rhythmic activity. It appears on the 6th or 7th day after fertilization, when the tentacles just begin to differentiate. The daily rhythmic activity in the polyp stage is most remarkably shown by day-contraction and night-expansion of the tentacles.

3. The endogenous nature of the daily rhythmic activity was studied by putting the young polyps in constant darkness on the 6th, 9th, 15th and 20th day respec tively after fertilization.

(i) The animals whose tentacles were differentiated in dark did not show any daily rhythmic activity.

(ii) The animals, whose tentacles were differentiated in the normal day-night condition and placed in constant darkness on the 9th day after fertilization (after living under normal day-night condition for 2 or 3 days, with developed tentacles), persisted about one daily rhythmic activity cycle only.

(iii) When the animals were placed in constant darkness on the 15th day after fertilization (after living under normal day-night condition for about a week, with developed tentacles), a stronger persistence of the daily rhythmic activity was observed (persistence was observed for 2 or 3 days in some case).

(iv) It was impossible to get further concrete results because of difficulty of rearing the young animals in healthy condition for more than 20 days after fertilization.

4. It can be concluded that, (i) for the emergence of the daily rhythmic activity of the sea-pen it is necessary to be triggered by the environmental rhythms after the accomplishment of differentiation of organs related to the daily rhythmic activity, and (ii) the endogenous nature is more and more firmly settled in accord with the progress of development.

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DISCUSSION

WERNER: Could you also investigate comparatively the development of the neural structures which are the morphological bases for the behavior?

MORI: No, I have not done.

CHENEY: Is the intensity of the contraction response modified by varying the wave length or spectra of light?

MORI: We are sorry we did not perform any kind of experiment to see the effect of light of various wave lengths.

MÜLLER: When using the movement of tentacles as criterion it seems me quite clear that a rhythmicity can not be observed until tentacles arise. I think you should use another criterion, too, in order to follow the development of rhythmicity. Did you?

MORI: Yes, we did. We observed carefully other features of behavior, such as direction of peristalsis or speed of swimming or creeping, but we could not find out any sign of overt daily rhythmic activity except the behavior of tentacles.