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Environmental Control of Gonadal Maturation in Laboratory-Reared Sea Urchins, *Anthocidaris crassispina* and *Hemicentrotus pulcherrimus*

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ABSTRACT—Gametogenic processes in laboratory-reared sea urchins, *Anthocidaris crassipina* and *Hemicentrotus pulcherrimus*, were compared under different experimental environments. The juvenile sea urchins were obtained by artificial fertilization. After preparatory rearing for 3 months under continuous illumination at 20°C, the juveniles were divided into groups and kept under various light and temperature regimes. The stages in gonadal maturation were determined according to a histological standard. In *A. crassispina* kept at ambient temperatures, the gonads matured under both continuous light (LL) and continous darkness (DD) simulataneously with those under ambient light (control). In *A. crassispina* kept under LL, the gonads matured at either constant 20 or 25°C earlier than those in the control but remained immature at 15°C. In *H. pulcherrimus* kept under LL, the gonads remained immature as long as the animals were kept at a constant temperature (15, 20, 25°C). If the water temperature was lowered stepwise from 25°C to 20°C and after a month to 15°C, the gonads matured about 3 months after the first temperature drop. The gonads of the fully grown sea urchins that had once experienced gonadal maturation in the outdoor tank showed the same response as those of the juveniles to the stepwise temperature drops. It is conceluded from previous [18] and the present results that in *A. crassispina, H. pulcherrimus* and *Pseudocentrotus depressus*, the three sea urchin species common to shallow water along the Japanese coast, the environmental factor determining their breeding season is not light but water temperature.

INTRODUCTION

Most marine invertebrates living in the temperate zone generally show species-specific annual reproductive cycles. In field studies of many sea urchin species [1–11], various environmental factors such as photoperiods, temperatures, food supplies and the lunar cycle have been presumed to serve as environmental cues to synchronize their reproductive cycles. To know the actual environmental factor regulating the reproductive activity in the sea urchin, it is necessary to examine gonadal responses to artificially manipulated environments under which animals were kept for a long time. Such efforts have been made mostly in *Strongylocentrotus purpuratus* [12–17]. Pearse and his colleagues have recently shown that the gametogenesis in that species is under photoperiodic control [16, 17].

We have started a series of long-term experiments on environmental control of gametogenesis in Japanese sea urchins. Since it has been reported in some echinoderms [13, 15, 22] that the annual reproductive calendar once set in the field persists long after the animals are transferred under experimetal conditions, we have used juvenile sea urchins produced by artificial fertilization and reared under a constant laboratory environment until they are transferred to experimental conditions. In the preceding paper [18], we have demonstrated in *Pseudocentrotus depressus* and *Hemicentrotus pulcherrimus* that the gonad matured independently of photic conditions. In this

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report, we present evidnece that light is not a main environmental factor regulating the gonadal activity also in *Anthocidaris crassispina* and further that the reproductive cycle is controlled by water temperature in *A. crassispina* and *H. pulcherrimus*.

MATERIALS AND METHODS

Adult sea urchins

Adults of *Anthocidaris crassispina* and *Hemicentrotus pulcherrimus* were collected in the intertidal zone near the Ushimado Marine Laboratory, Okayama Prefecture in July and March, respectively. The breeding seasons of these species at that place are from June to August and from January to March, respectively.

Obtaining juvenile sea urchins

Embryos used in each series of experiments were obtained by artificial fertilization of gametes spawned from one male and one female. They were reared according to the method by Kakuda [19–21] after slight modifications, the details of which were described in the preceding paper [18]; briefly, the swimming larvae were fed with a diatom, *Chaetoceros gracilis,* and the metamorphosis was induced by giving plastic plates with a film of diatoms attaching on both surfaces (about 10 days and 15 days after fertilization for *A. crassispina* and *H. pulcherrimus,* respectively). The juveniles were kept at 20°C under continuous illumination of the ceiling lights until they were transferred into experimental environments.

Maintenance of sea urchins under experimental environments

When the juveniles had grown into a certain size range (about 90 days after fertilization; the test diameter were 4–10 mm and 4–7 mm in *A. crassispina* and *H. pulcherrimus,* respectively), they were divided into groups matched for similar size distributions, placed in plastic cages (30 cm in diameter), and transferred into experimental environments. Except for the control groups, we used indoor aquaria (120×65×45 cm), each equipped with a system to recirculate constant temperature sea water. In the aquaria for constant temperature groups, the recirculating sea water at a constant temperature was continuously replaced little by little by new sea water. The aquaria for ambient temperature groups were continuously supplied with running sea water at the ambient temperatures. The illumination for continous light (LL) was given by some 40 W fluorescent bulbs (about 2000 lux). The aquaria for constant darkness (DD) were made light-tight using opaque plastic boards. The lid of the aquaria for DD was opened briefly (less than 15 min) every 3 or 4 days at an unfixed time of the day for feeding and/or cleaning. The control groups wer kept in an outdoor tank under the ambient light supplied continously with running sea water at ambient temperatures. During the expriments the sea urchins were provided with an unlimited amount of food. They were fed with *Ulva pertusa* from March to October and some species of *Sargassum* (*S. horneri, S. serratifolium, S. tortile* etc.) from November to February. Feces and sediments were removed from the aquaria at least once a week.

Sampling

The test diameters and wet weights of all animals were periodically measured. Some randomly selected animals were dissected periodically for histological observations. The gonads were weighed and pieces were fixed in Bouin's solution. Paraffin-embedded gonadal tissues were sectioned and stained with haematoxylin and eosin. Since exact measuring of wet gonad weights is difficult and the gonadal indices (percentage of wet gonad weight in wet animal weight) do not fully correlate with the degree of gonadal maturity in small and rapidly growing sea urchin, we judged the degree of gonadal maturity in the histogical sections according to the standard that was defined in the preceding paper [18] as shown schematically in Figure 1.

RESULTS

Anthocidaris crassispina

Figure 2 shows the results in the individuals derived from the zygotes fertilized on 7 July 1986

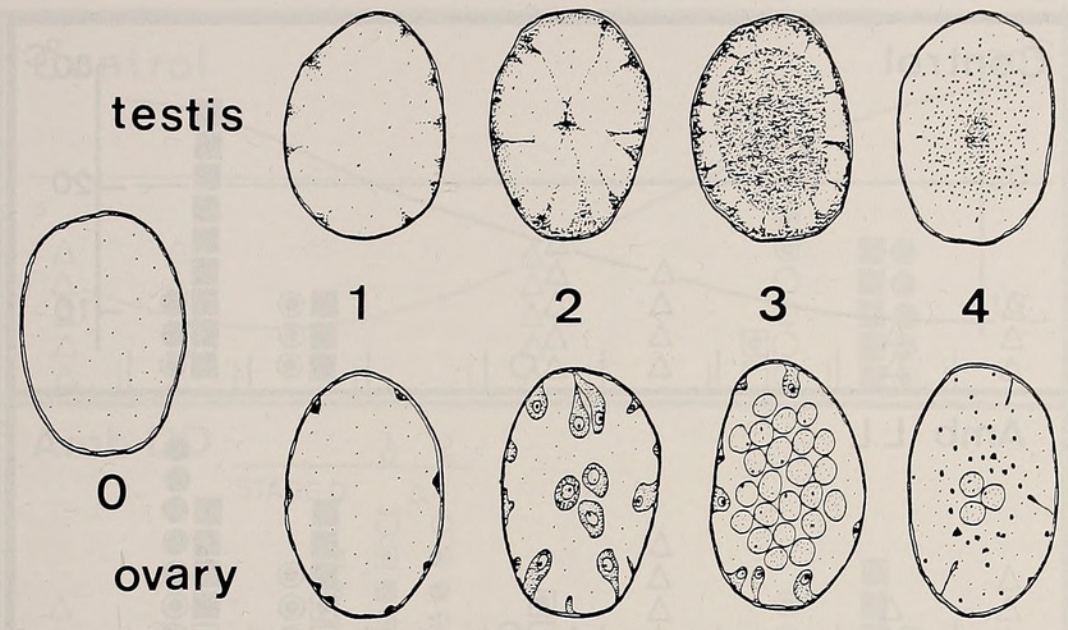


FIG. 1. Schematic representation of stages in the development of the sea urchin gonad.
Stage 0: No obvious germ cells are found and the gonadal sexes cannot be identified in the section.
Stage 1: *Ovary*—A few small oocytes are present in the periphery of the ovarian lobe. No large oocytes are found.
Testis—Small clusters of spermatogenic cells are present in the periphery of the testicular lobe.
Stage 2: *Ovary*—Many large oocytes with a prominent germinal vesicle are present in the ovarian wall. Some oocytes migrate toward the center of the ovarian lobe. No mature eggs are present.
Testis—The wall of the testicular lobe is lined with columns of spermatocytes. Small masses of spermatozoa are present in the center of the testicular lobe.
Stage 3: *Ovary*—Numerous mature eggs are present in the center of the ovarian lobe (ovarian cavity).
Testis—The space in the center of the testicular lobe (lumen) is filled with large numbers of spermatozoa.
Stage 4: *Ovary*—The ovarian cavity contains only a few relict eggs. Oocytes are few in the ovarian wall.
Testis—The lumen is almost empty with a few relict spermatozoa. Spermatogenic cells are few in the testicular wall.

and transferred into the experimental conditions on 24 September 1986. We tried to compare the processes of gonadal maturation among groups maintained under the following 6 environmental conditions: continuous light (LL), continuous darkness (DD) and ambient light (control) each at ambient temperatures, and constant 15°C, 20°C and 25°C each under LL. Since we had accidentally lost the group under DD at ambient temperatures, we partly repeated the experiments in 1987. In the repeated experiments, the juveniles derived from the zygotes fertilized on 9 July 1987 were placed under DD and ambient light (control) each at ambient temperatures on 22 September 1987. The results are shown in Figure 3. At the beginning of both series of experiments, the gonads were rudimentary and the sexes were unidentifiable (stage 0) in each individual.

In the controls, the gonads reached the full maturity (stage 3) about one year after fertilization, a little earlier in 1986 than in 1987 (Figs. 2 and 3). In the groups kept under LL and DD at ambient temperatures, the gonads reached stage 3 at the same time as those in each control (Figs. 2 and 3). In all the three groups, the testes became ripe earlier than the overies.

In the groups kept at constant 20 and 25°C under LL, the gonads grew more rapidly and became mature about 3 months earlier than those in the control (Fig. 2). Although individuals kept at 25°C were unfortunately biased to male, there seems to be little difference in the rate of gonadal maturation between the groups kept at 20 and 25°C. In the group ket at 15°C under LL, no individuals

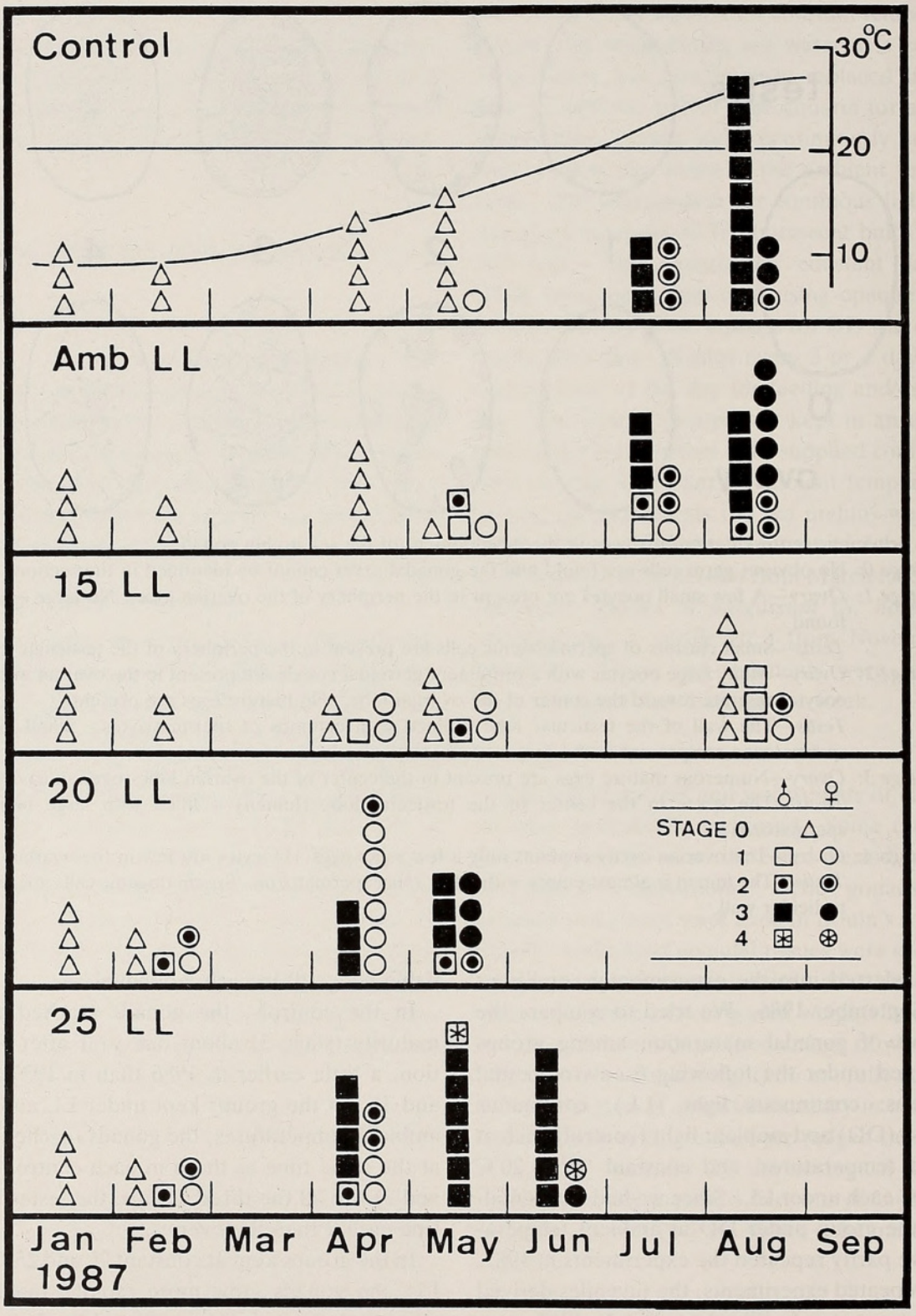


FIG. 2. The stages of the gonads of *Anthocidaris crassispina*. Each symbol represents one individual. Sea urchins were reared from zygotes of the same batch fertilized on 7 June 1986 and were kept under constant illumination at 20°C until they were transferred on September 1986 to one of the following experimental environments: ambient temperatures under ambient light (Control), and ambient temperatures (Amb LL), constant 15°C (15 LL), 20°C (20 LL) and 25°C (25 LL) under continuous light. The change in sea water temperature near the Ushimado Marine Laboratory is shown at the top.

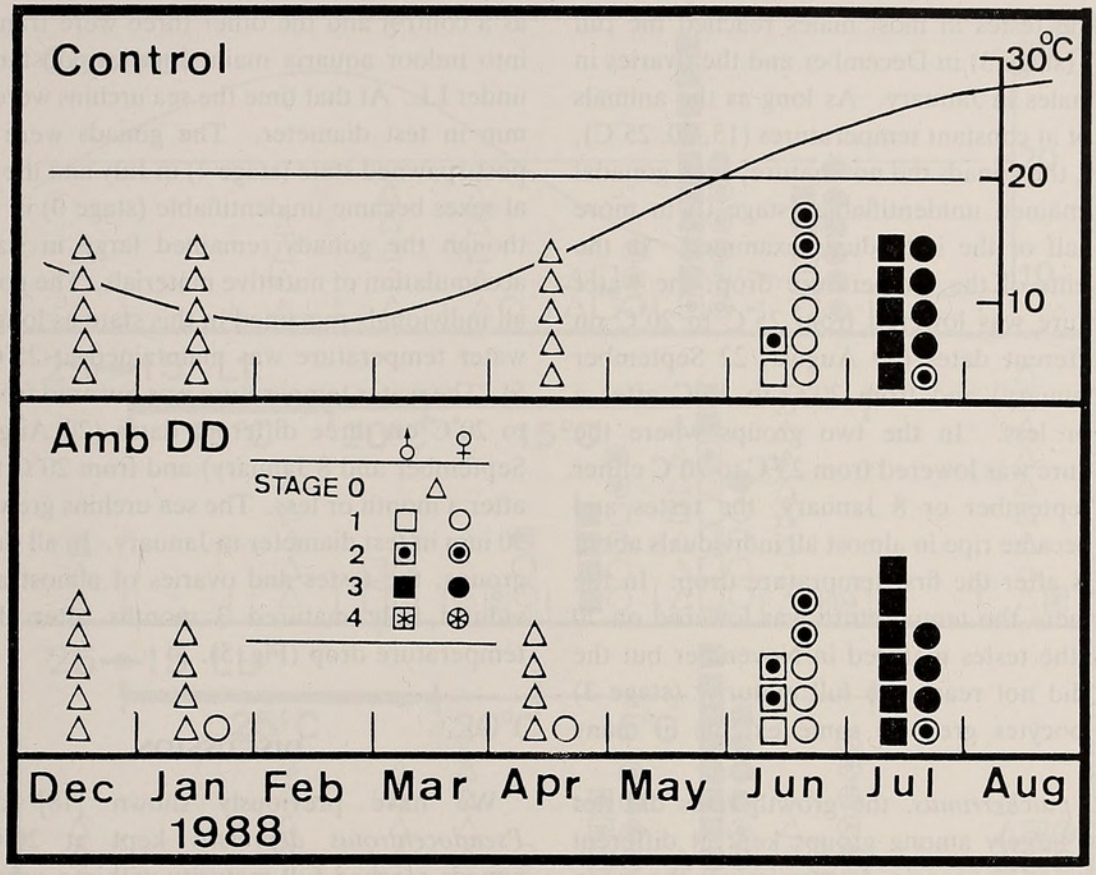


FIG. 3. The stages of the gonads of *Antohocidaris crassispina*. Each symbol represents one individual. Sea urchins were reared from zygotes of the same batch fertilized on 9 June 1987 and were kept under constant illumination at 20°C until they were transferred to ambient light at ambient temperatures (control) or to continuous darkness at ambient temperatures (Amb DD) on 22 September 1987. The change in sea water temperature near the Ushimado Marine Laboratory is shown at the top.

reached stage 3 and in a half of the individuals examined the gonadal sexes remained unidentifiable (stage 0) when the gonads in the control had matured (Fig. 2).

There was little difference in the growth rates among the two control groups and the groups kept under LL and DD at ambient temperature; mean test diameters one year after fertilization were 20–22 mm. The groups kept at 20 and 25°C grew more rapidly and the one kept at 15°C more slowly than the control; mean test diameters one year after fertilization were 31, 28 and 20 mm in 25, 20 and 15°C groups, respectively.

Hemicentrotus pulcherrimus

In the preceding paper [18] we have reported that in *H. pulcherrimus*, the gonads matured under LL as well as DD when the animals were kept at ambient temperatures but remained immature at constnat 20°C irrespective of the photic conditions tested. In the present experiments we examined the effects of three constant temperatures (15, 20 and 25°C) and a temperature drop from 25°C to 15°C on the gonadal maturation. In the experiments of the temperature drop, the water temperature was lowered stepwise from 25°C to 20°C and after a month to 15°C since the direct drop from 25°C to 15°C seemed harmful to the sea urchins. Except for the control groups that were kept under ambient light at ambient temperatures, all the experimental groups were maintained under LL.

Figure 4 shows the results of the experiment using the juveniles derived from zygotes fertilized on 22 March 1987 and transferred into the experimental conditions on 30 June 1987. At the beginning of the experiment animals were 4–7 mm in test diameter, the gonads were small and the sexes were unidentifiable (stage 0). In the control

group, the testes in most males reached the full maturity (stage 3) in December and the ovaries in most females in January. As long as the animals were kept at constant temperatures (15, 20, 25°C), however, the gonads did not mature; even gonadal sexes remained unidentifiable (stage 0) in more than a half of the individuals examined. In the experiments of the temperature drop, the water temperature was lowered from 25°C to 20°C on three different dates (20 August, 22 September and 8 January) and from 20°C to 15°C after a month or less. In the two groups where the temperature was lowered from 25°C to 20°C either on 22 September or 8 January, the testes and overies became ripe in almost all individuals about 3 months after the first temprature drop. In the group where the temperature was lowered on 20 August, the testes matured in November but the ovaries did not reach the full maturity (stage 3) though oocytes grew to some extents in many ovaries.

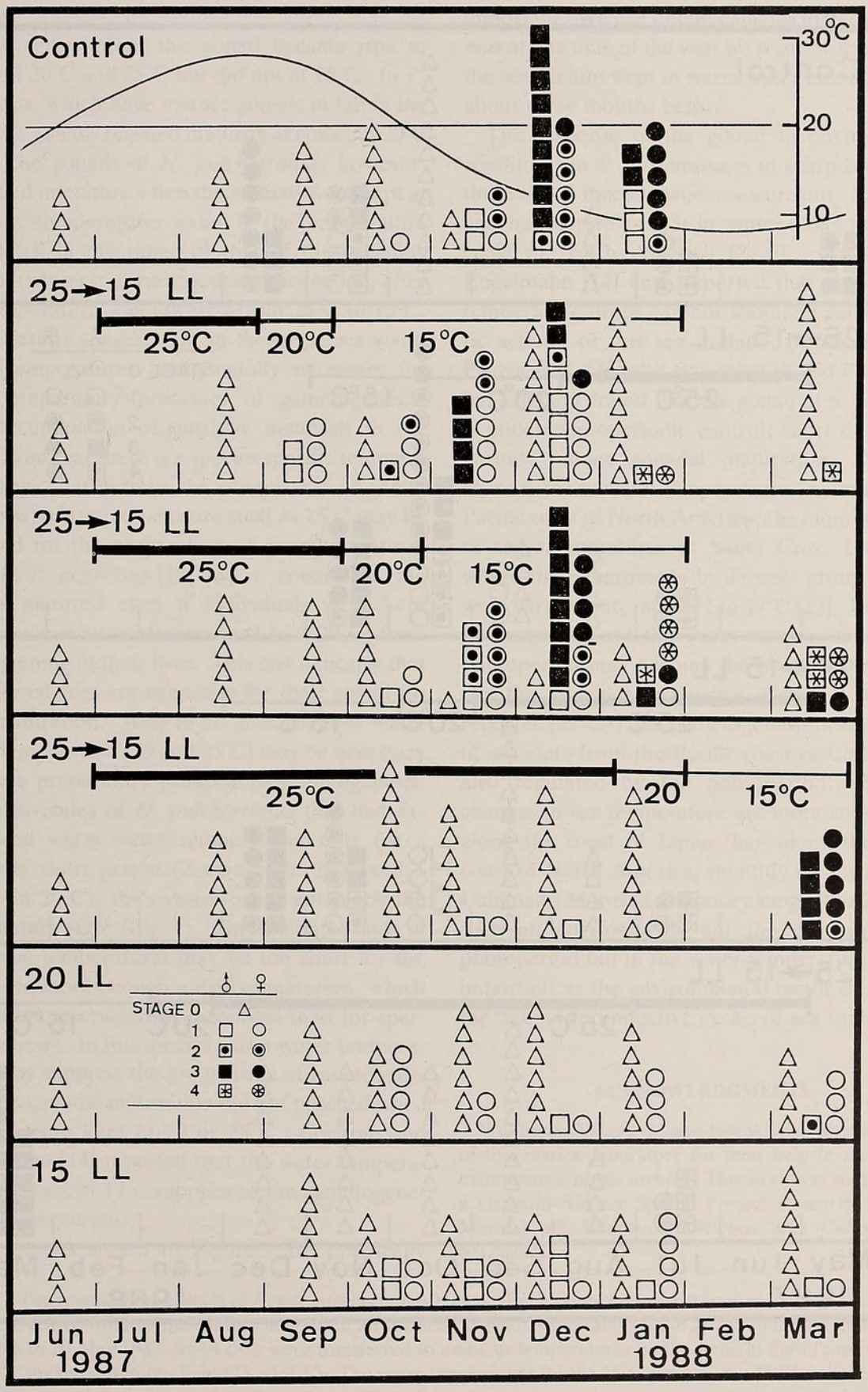
In *H. pulcherrimus*, the growth rates did not differ so largely among groups kept at different temperatures as seen in *A. crassispina*; the mean test diameters in January (10 months after fertilization) were 21, 23 18 and 24 mm in 15, 20, 25°C groups and the control (ambient temperatures), respectively.

We examined whether or not the gonads in fully grown sea urchins were also responsive to the temperature drop using individuals that had once experienced gonadal maturation in the outdoor tank. The sea urchins were derived from the zygotes fertilized on 29 March 1986 and reared at 20°C under continuous illumination until they were transferred on 21 June 1986 into outdoor tanks that was supplied with running sea water of ambient temperatures. Their gonads were in the mature state (stage 3) from February to May 1987. On 20 May 1987, the sea urchins were divided into four groups; one was remained in the outdoor tank as a control and the other three were transferred into indoor aquaria maintained at constant 25°C under LL. At that time the sea urchins were 23–25 mm in test diameter. The gonads were in the post-spawned state (stage 4) in July and the gonadal sexes became unidentifiable (stage 0) in August though the gonads remained large in size with accumulation of nutritive materials. The gonads in all individuals remained in this state as long as the water temperature was maintained at 25°C (Fig. 5). The water temperature was lowered from 25°C to 20°C on three different dates (20 August, 22 September and 8 January) and from 20°C to 15°C after a month or less. The sea urchins grew to 27–30 mm in test diameter in January. In all the three groups, the testes and ovaries of almost all individuals fully matured 3 months after the first temperature drop (Fig. 5).

DISCUSSION

We have previously shown [18] that in *Pseudocentrotus depressus* kept at 20°C, the gonads reached full maturity within a year under all the photic conditions tested (LL, DD, and in-phase and out-of-phase photoperiods) and that in *Hemicentrotus pulcherrimus* kept under LL and DD at ambient temperatures, the gonads matured simultaneously with those of the control animals kept under the ambient photoperiod at ambient temperatures. In the present experiments, the gonads of *H. pulcherrimus* matured under LL after stepwise drops of temperature from 25°C to 15°C. In contrast to these two species, *Anthocidaris crassispina* has the breeding season in summer. In this species also, the gonads matured under DD as well as LL simultaneously with those of the control animals kept under the ambient photoperiod. These results strongly suggest that the photoperiod is not the environmental factor controlling the annual reproductive cycle in these three sea urchin

FIG. 4. The stages of the gonads of *Hemicentrotus pulcherrimus*. Each symbol represents one individual. Sea urchins were reared from zygotes of the same batch fertilized on 22 March 1987 and were kept under constant illumination at 20°C until they were transferred on 30 June 1987 to one of the following experimental conditions: ambient temperatures under ambient light (control), and constant 25°C (25→15 LL), 20°C (20 LL) and 15°C (15 LL) under continuous light. The water temperature for the 25°C groups (25→15 LL) were lowered to 15°C via a short interval of 20°C on three different dates. The change in sea water temperature near the Ushimado Marine Laboratory is shown at the top.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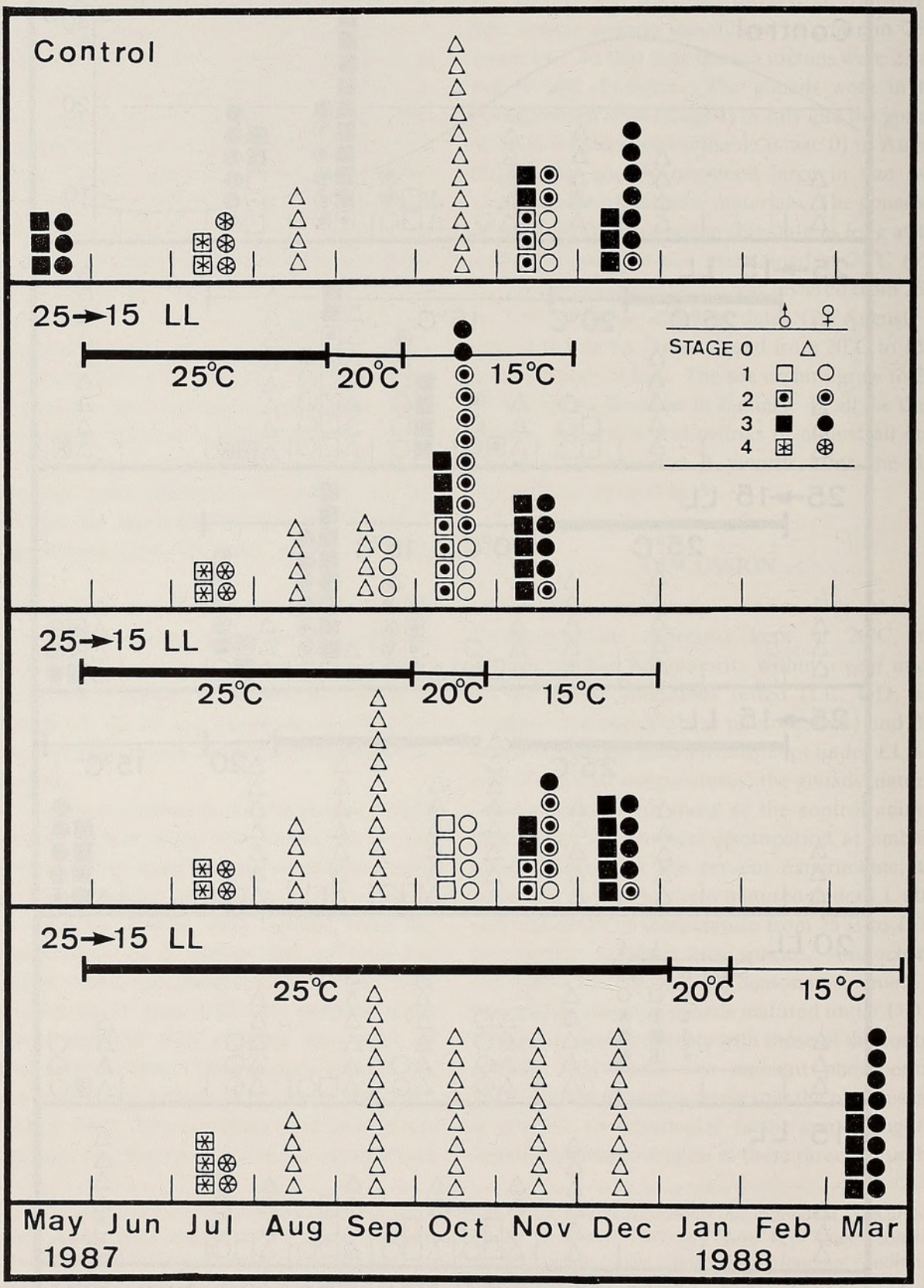
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species.

In *A. crassispina,* the gonad became ripe at constant 20°C and 25°C but did not at 15°C. In *P. depressus,* which have mature gonads in fall in the field, the gonads reached maturity at constant 20°C [18]. The gonads of *H. pulcherrimus,* however, remained immature when the animals were kept at constant temperatures even if the temperature (15°C) fell in the range of that of the breeding season (winter); gametogenesis proceeded after the temperature was lowered from 25°C to 15°C. These results suggest that in these species warm water temperatures are generally necessary for some preparatory processes of gametogenesis, e.g., accumulation of nutritive materials in the gonad, and that there is a species specific temperature range that permits the progression of gametogenesis; water temperature such as 15°C may be too cold for the preparation of gonadal maturation. In *P. depressus* [18] and *A. crassispina,* the gonads matured even if individuals were kept under a constant environment (LL at 20°C) from the beginning of their lives. This fact indicates that no external cues are necessary for their gonads to start maturation. Also in *H. pulcherrimus* warm water temperature (20 and 25°C) may be necessary for some preparatory processe of gametogenesis. In the juveniles of *H. pulcherrimus* that had experienced warm water temperatures only for a relatively short period (2 months in 25°C and 1 month in 20°C), the ovaries could not reach the fully mature state (Fig. 4). Three months' stay in the warm temperatures may be too short for the ovaries to store enough nutritive materials, which may be more crucial for oogenesis than for spermatogenesis. In this species warm water temperatures may suppress the progression of ganetogenesis. The gonadal maturation did not proceed when animals were kept in 20 or 25°C. Cochran and Engelmann [14] reported that the water temperature in excess of 17°C suppressed the gametogenesis in *S. purpuratus.*

The results shown in Figure 5 are practically important. We can obtain gametes of *H. pulcherrimus* at any time of the year we want by transferring the sea urchins kept in warm water into cold water about three months before.

The response of the gonad to environmental conditions in *S. purpuratus* is in sharp contrast to those in our three Japanese sea urchins. *S. purpuratus* has mature gonads in winter along the Pacific coast of North America [2, 5]. Cochran and Engelmann [14] have reported that experimental temperature drops can not stimulate gametogenetic activity of this sea urchin. Boolootian [12], Pearse *et al.* [16] and Bay-schmith and Pearse [17] have demonstrated that the gonad of *S. purpurtus* is under photoperiodic control; short day photoperiods induce gonadal maturation. Seasonal changes in sea water temperatures are small on the Pacific coast of North America; the monthly means of sea temperatures at Santa Cruz, California, where the experiments by Pearse' group [16, 17] were carried out, range 12 to 17°C [22]. In such an environment, not the water temperature but the photoperiod may become the main factor to control the annual reproductive cycle. It has been reported [22–24] that gametogenesis in two species of sea stars from the Pacific coast of California is also regulated by the photoperiod. Seasonal changes in sea temperature are more conspicuous along the coast of Japan than along the Pacific coast of North America; monthly means near the Ushimado Marine Laboratory range 9 to 26°C. In such an environment, not the changes in the photoperiod but in the water temperature may be important as the environmental factor to regulate the annual reproductive cycles of sea urchins.

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FIG. 5. The stages of the gonads of *Hemicentrotus pulcherrimus.* Each symbol represents one individual. Sea urchins were reared from zygotes of the same batch fertilized on 29 March 1986 and kept in an outdoor tank from 30 June 1986 to 20 May 1987, when they were transferred to ambient temperatures under ambient light (comtrol) or to 25°C under continuous light (25→15LL). The water temperature for the 25°C groups (25→15 LL) was lowered to 15°C via a short interval of 20°C on three different dates.

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