

## BROODSTOCK CONDITIONING IN THE BASKET COCKLE, *CLINOCARDIUM NUTTALLII*

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**ABSTRACT** Factors affecting broodstock conditioning in the hermaphroditic basket cockle, *Clinocardium nuttallii*, were examined in laboratory experiments. Although a 13-wk experiment conducted at 16°C failed to bring *C. nuttallii* broodstock into spawning condition under different dietary treatments, initiation of gametogenesis was apparent for those broodstock maintained at 2.5°C. Condition indices were significantly greater when broodstock were fed a combination of *Isochrysis galbana* (Tahitian strain, T-iso) and *Chaetoceros gracilis* (CG) or T-iso and *Thalassiosira pseudonana* (3H) than when fed a combination of *Tetraselmis suecica* (TS) and 3H or TS and CG, indicating that T-iso may be a better food source than TS for cockle broodstock conditioning.

**KEY WORDS:** broodstock, *Clinocardium nuttallii*, cockle, conditioning, microalgae, temperature

### INTRODUCTION

Broodstock conditioning is a major step in the operation of bivalve hatcheries. The maturation of broodstock is controlled by both endogenous and exogenous factors, and among the latter factors, temperature and food are the most important (Sastry 1968, Bayne et al. 1976, Mann 1979, Newell et al. 1982, Muranaka & Lannan 1984, Barber & Blake 2006). It has been shown that the development rate of gonads and conditioning time in molluscs are directly related to water temperature (Mann 1979, Muranaka & Lannan 1984). This has allowed the development of a useful quantitative technique, which is based on the concept of effective accumulative temperature (EAT) or cumulative difference between the holding temperature and the theoretical temperature below which the development ceases, that is, the biological zero point (BZP). The technique provides an effective management tool to predict the time required for broodstock to reach spawning condition during hatchery production (Kikuchi & Uki 1974a, Kikuchi & Uki 1974b, Mann 1979).

In addition to temperature, the quality of food offered to molluscan broodstock has been identified as having a major influence on their reproductive performance and on the quality of eggs and larvae produced (Robinson 1992a, Robinson 1992b, Farias & Uriarte 2001, Uriarte et al. 2004). Millican and Helm (1994) reported that fecundity and larval survival of the oyster *Ostrea edulis* were higher when the broodstock were given the diets containing the microalga *Isochrysis galbana* (Tahitian strain, T-iso). Similarly, in the scallop *Pecten maximus*, Soudant et al. (1996a, 1996b) found that maturity of the broodstock and hatch rate of the eggs to D-larvae were improved on a diet of T-iso. In the scallop *Mimachlamys asperrima*, O'Connor et al. (2000) observed that fecundity was greatest when the broodstock were fed *Chaetoceros gracilis* (CG). Dunphy et al. (2006) suggested that difficulties in the broodstock culture of the oyster

*O. chilensis* might be caused by inappropriate microalgal diets. It is, therefore, advantageous to identify the dietary items that promote the maximum performance of the broodstock for hatchery production of a particular target species.

The basket cockle, *Clinocardium nuttallii*, is found along the Pacific coast of North America from Alaska to San Diego (California), with a disjunct population in Hokkaido, Japan (Coan et al. 2000). In the province of British Columbia (BC), Canada, there has been a recent interest in the commercial cultivation of this species because of its relatively fast growth, its ability to utilize various substrates from silt/clay to coarse sand, and its adaptation to survive, grow, and reproduce in the cold waters of the Pacific Ocean coast of BC and Alaska (Gallucci & Gallucci 1982, Coan et al. 2000). However, very little published information is available on the requirements for successful commercial cultivation of *C. nuttallii*. As a first step towards generating such information, this study is aimed at examining the effect of microalgal diets and temperature on the broodstock conditioning of *C. nuttallii*.

### MATERIALS AND METHODS

#### *Collection and Maintenance of Cockles*

Ripe adult *C. nuttallii* with a mean ( $\pm$ SD) shell length of 61.5  $\pm$  5.1 mm ( $n = 100$ ) were collected in June 2006 from a shellfish farm site (48°58'20"N, 123°41'27"W) in the Strait of Georgia (BC Fishery Management Area 14), transported to the Center for Shellfish Research (CSR) at Malaspina University-College, and placed in a round, 430-L flat-bottom tank supplied with a continuous flow of recirculated seawater at a temperature of 15°C–16°C and a salinity of 32.0‰–33.0‰. The cockles started spawning after overnight maintenance without further intervention. These animals were maintained in the system for a further eight-week period, during which they were fed daily with 200 L of a mixture of the microalgae T-iso, *Thalassiosira pseudonana* (3H), *Tetraselmis suecica* (TS), and CG. Prior to the conditioning experiment, the cockles were induced to spawn

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by adding excessive amounts of algae to the holding tank. Natural reproductive cycles of the cockles from the collection site in the present study are not yet clear. However, Gallucci and Gallucci (1982) found that in Washington State, USA, natural spawning of cockles took place from April to November, and gametogenesis might be initiated in any months of the year except July, August, and September.

#### Effect of Dietary Items on Broodstock Conditioning at Ambient Temperature

Fifty cockles were randomly counted into each of five elliptical fiberglass tanks containing 130 L of seawater. To facilitate separation of fecal material from the cockles, two perforated plastic trays (each with a base area of  $44 \times 31 \text{ cm}^2$  and 25 cockles) were positioned in each tank, 10 cm above the tank bottom. The tanks were provided with seawater at a flow rate of  $1.5 \text{ L min}^{-1}$  and moderate aeration, and water depth maintained at 30 cm with standpipes. Seawater was filtered through one  $5\text{-}\mu\text{m}$  and two  $0.35\text{-}\mu\text{m}$  cartridge filters and the temperature maintained at  $16^\circ\text{C}$  ( $\pm\text{SD} = 1^\circ\text{C}$ ,  $n = 130$ ) throughout the experiment.

The five holding tanks were each used in a different dietary treatment: (1) binary combination of T-iso and 3H (T-iso/3H), (2) T-iso and CG (T-iso/CG), (3) TS and 3H (TS/3H), (4) TS and CG (TS/CG), and (5) a starved control. Previously, we showed that optimal daily food ration, in terms of best absorption rate for adult cockles fed T-iso at  $14^\circ\text{C}$ , was about 5% of their dry-tissue weight (DTW). Therefore in the present study, energy content in the amount of T-iso, which is equivalent to 5% of the initial DTW of the cockles, or 2.44 kJ for a single cockle with an average DTW of 2.61 g, was considered as the daily reference food energy for each dietary treatment and split equally between the two microalgal species. This amount of energy (1.22 kJ) was then converted to the equivalent number

of cells (daily feeding amount), based on the cellular energy content (Whyte 1987) and the cellular dry weight (the present study) for each microalga. Detailed energy constituents, phytoplankton cellular dry weights, and daily feeding amounts for each dietary treatment are presented in Table 1. Products of the calculated feeding values and the numbers of cockles remaining in each tank hence gave the total daily feeding amounts for each microalgal treatment. The daily rations were metered to each tank over a 20-h period. The tanks were drained and flushed daily and scrubbed weekly. A photoperiod of 12-h light and 12-h dark was maintained using two 40-W fluorescent lights placed 2.5 m above the tanks. The experiment was carried out for a period of 13 wk. We were aware of the possibility of tank effects on experimental results caused by the lack of replication of mass-culture units. Precautions were therefore put in place, which entailed strict application of similar management practices across all experimental tanks.

#### Cold Temperature (BZP) Treatment

An additional experiment was set up at the same time to examine if there was any gonadal development when the cockles were held at cold temperatures close to the BZP ( $2.8^\circ\text{C}$ – $2.9^\circ\text{C}$ ) for cockle larval development (Liu et al. 2008). A subsample of 25 cockles, from the same common batch of animals used for the above dietary experiment, was put into a holding tank containing 60 L seawater and a single perforated plastic tray suspended off the bottom, as described previously. Water temperature was then decreased rapidly from the ambient  $16^\circ\text{C}$  to the experimental temperature of  $2.5^\circ\text{C}$  ( $\pm\text{SD} = 0.5^\circ\text{C}$ ,  $n = 130$ ) at a rate of  $1^\circ\text{C h}^{-1}$ . The cockles received seawater at a flow rate of  $0.25 \text{ L min}^{-1}$  and were fed a diet of T-iso and CG mixed in a ratio of 1:1 by energetic content to give 0.24 kJ per cockle per day (10% of the daily reference energy as used in the conditioning experiment). Other experimental conditions—including

TABLE 1.  
Energy constituents and daily feeding amounts of microalgae for an average-sized cockle *Clinocardium nuttallii* in different dietary treatments.

Mean Initial SL (mm)	Mean Initial DTW (g)	5% Initial DTW (g)	Reference Daily Food Energy in Each Treatment <sup>1</sup> (kJ per average-sized cockle)	
61.5	2.61	0.131	2.44 (energy content in 0.131 g T-iso)	
Dietary Treatments	Daily Energy Constituents (kJ per microalga per cockle)	Daily Feeding Amount		
		g Algae per Cockle <sup>1</sup>	Cells ( $\times 10^9$ ) per Cockle <sup>2</sup>	
T-iso/3H	T-iso	1.22	0.065	3.42
	3H	1.22	0.106	3.62
T-iso/CG	T-iso	1.22	0.065	3.42
	CG	1.22	0.113	1.80
TS/3H	TS	1.22	0.094	0.50
	3H	1.22	0.106	3.62
TS/CG	TS	1.22	0.094	0.50
	CG	1.22	0.113	1.80
Starved control	—	—	—	—

<sup>1</sup> Calculation based on energy contents of 18.7, 11.6, 13.0, and 10.8 kJ g<sup>-1</sup> for T-iso, 3H, TS, and CG, respectively (Whyte 1987).

<sup>2</sup> Calculation based on cellular dry weights of 19.1, 29.2, 190.0, and 62.8 pg cell<sup>-1</sup> for T-iso, 3H, TS, and CG, respectively (this study). T-iso: *Isochrysis galbana* (Tahitian strain), 3H: *Thalassiosira pseudonana*, TS: *Tetraselmis suecica*, CG: *Chaetoceros gracilis*, SL: cockle shell length, DTW: cockle dry tissue weight.

the photoperiod regime, tank cleaning procedures, and duration of the experiments—were similar between the two experiments.

### Sampling

Samples for histological examinations of the gonads were taken at the start of the experiments and then at 2, 4, 6, 8, 10, 12, and 13 wk after the initial stocking for the conditioning experiment, and at 2, 6, 10, and 13 wk for the experiment with the BZP treatment. Initial gonadal conditions were examined in six cockles sampled at the start of the experiments. Subsequently, at each sampling time, four cockles (two from both trays) were removed from each tank and for each animal, the upper part of the foot (including the visceral mass) was excised, fixed in Davidson's fixative, and stored in 70% ethanol. Samples were later processed by conventional histology and stained with haematoxylin and eosin. For each sample, two 5- $\mu$ m thick cross sections were made, one through the digestive gland and the other through the middle of the upper part of the foot. The specimens were examined for determination of gonad maturation following the general scheme described for *C. nuttallii* by Gallucci and Gallucci (1982): Grade 1: initiation of gametogenesis; Grade 2: developing gametes; Grade 3: ripe; Grade 4: recently spawned; and Grade 5: postspawning recovery. Where applicable, the diameters of young oocytes (early vitellogenic oocytes) were measured using the software Image-Pro Plus for Windows (Media Cybernetics Inc., MD, USA), from 10 randomly chosen cells in each replicate cockle.

At the start and the end of the conditioning experiment, an additional six individuals were also sampled from each treatment for the determination of condition index (CI):

$$CI = (\text{dry tissue weight} / \text{total dry weight}) \times 100$$

Cockle dry weight was obtained by drying the animals at 60°C to constant weight. Because of the high cumulative mortality, however, sampling for CI was not possible for the starved control at the end of the experiment.

To determine the cellular dry weight of the microalgae, known volumes and concentrations of the algal suspensions were centrifuged for 10 min at 4,000 rpm at 4°C. The pellets were rinsed and resuspended in 0.5 M ammonium formate to remove salts, recentrifuged for 5 min, and then dried at 60°C to constant weight.

### Statistical Analysis

The effect of dietary treatment on condition index was examined with one-way ANOVA, followed by a Student-Newman-Keuls multiple range test to detect which means differed significantly ( $P < 0.05$ ). Data were square-root transformed to remove heterogeneity of variances as confirmed by Levene's Test, and normality of transformed data was confirmed by Kolmogorov-Smirnov Test. All statistics were conducted using the statistical software NCSS (Kaysville, UT, USA).

## RESULTS

During the 13 wk of the conditioning experiment at 16°C, gametogenic activities of *C. nuttallii* were essentially absent in all dietary treatments and the starved control. The initial gonads contained small, irregularly-shaped acini, which were

infrequently distributed between the digestive gland and the muscular layer of the visceral mass as well as the areas lateral to the intestinal loops and between the muscles extending to the lower part of the foot. The lumens of the acini examined at this stage were either filled with hemocytes or with lytic oocytes, or empty (Fig. 1A,B). In addition, the inner walls of the acini were occasionally dispersed with strongly basophilic stem cells (nucleoli), and the interacinal area was invaded by hemocytes with no abundant connective tissues in between (Fig. 1A). These gonadal stages were similar to those of recently spawned or post spawning-recovery, as classified by Gallucci and Gallucci (1982) for this species. The gonadal features as described in Figure 1(A,B) did not change perceptibly in most of the specimens examined throughout the conditioning period. Of the total number of 140 cockles inspected during the entire experiment, only four were found to have mature oocytes and spermatozoa present in their acini (Fig. 1C) and 11 others to have spermatozoa present only occasionally in their acini, and these observations were not made from a same time sampling point nor from a same dietary treatment.

In contrast, when the cockles were kept at 2.5°C, as in the BZP treatment, developing (growing) oocytes were apparent in all eight specimens examined 10 and 13 wk after the onset of the experiment. On average, about one-third of the acini for each cockle examined were observed to be filled with young oocytes (Fig. 1D), with a mean diameter of 17.7  $\mu$ m ( $\pm$ SD = 2.7  $\mu$ m,  $n = 80$ ).

When T-iso was included in the diet for the broodstock, their condition indices increased significantly ( $P < 0.05$ ) from 7.2% initially to 10.0% for the T-iso/3H and to 10.2% for the T-iso/CG treatments at the end of the 13-wk conditioning period (Fig. 2). These results were significantly ( $P < 0.05$ ) higher than those obtained when TS was included in the diets (3.3% and 5.1% for the TS/3H and TS/CG treatments, respectively, at the end of the trial). There was also a significant ( $P < 0.05$ ) difference in the condition indices between the two diets with TS (Fig. 2). Apart from a much smaller foot muscle, the cockles fed the two diets with TS had a smaller, tan-colored digestive gland compared with the larger, dark-colored digestive gland of their counterparts fed the diets with T-iso.

## DISCUSSION

Techniques used in bivalve hatcheries for conditioning broodstock outside normal spawning seasons are generally similar among species. These basically consist of maintaining the adults (collected from the wild) at elevated temperatures and providing them with adequate food items and rations (Utting & Spencer 1991, Utting & Millican 1997, Gérard & Robert 1999). The application of these standard techniques, however, did not bring about mature gonads in *C. nuttallii* over a 13-wk conditioning period with the use of different diets. Because previously we had succeeded in conditioning batches of *C. nuttallii* broodstock at the CSR with similar husbandry techniques, failure in the conditioning attempt in the present study is not likely to be a result of suboptimal environments (e.g., temperature, salinity, and photoperiod) and inadequate feeding regime as a consequence of poor management. The increased condition indices obtained at the end of the experiment for *C. nuttallii* fed the two diets with T-iso simply reveal that the animals did not use available energy sources to fuel

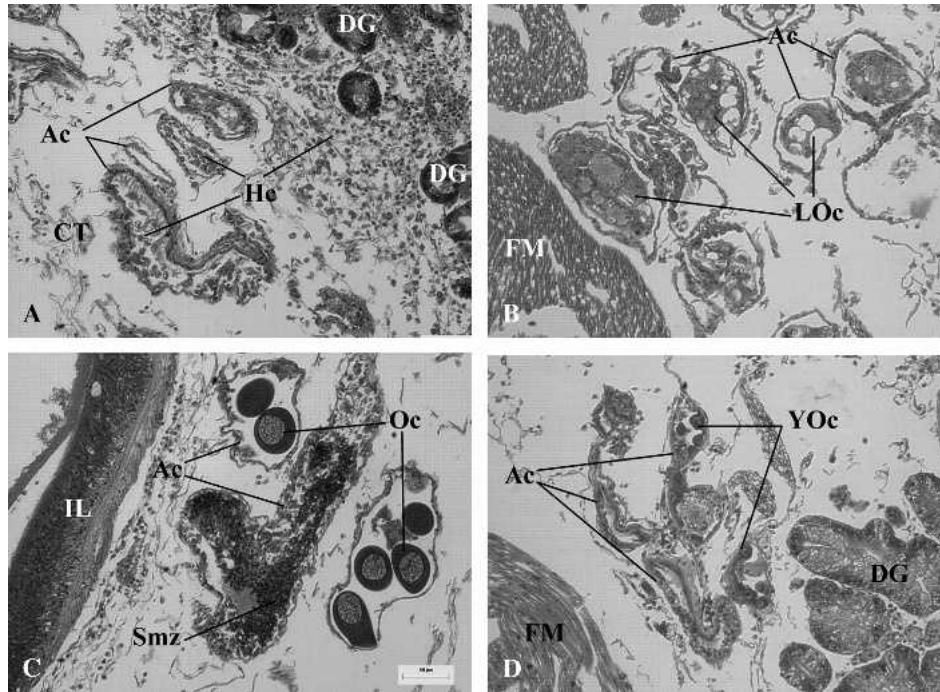


Figure 1. Histological details of the gonads in *Clinocardium nuttallii* held at 16°C (A–C) and 2.5°C (D) for a 13-wk period. Ac: acinus, CT: connective tissue, DG: digestive gland, FM: foot muscle, Hc: hemocytes, IL: intestinal loop, LOc: lytic oocytes, Oc: oocytes, Smz: spermatozoa, YOc: young oocytes. See text for complete description. Bar = 50  $\mu$ m.

reproduction. Therefore, failure in the conditioning process requires a search for other potential reasons (see later for a possible explanation).

The initial gonadal condition of broodstock has been shown to play a significant role in determining the time needed for bivalves to reach gonadal maturation and even the conditioning success (Robinson 1992c, Chávez-Villaba et al. 2002). Muranaka and Lannan (1984) found that, for sexually undifferentiated oysters *Crassostrea gigas* with gonads containing no gametes, mature oocytes were not noticed until 14–56 days after the conditioning experiment was started (16°C–22°C).

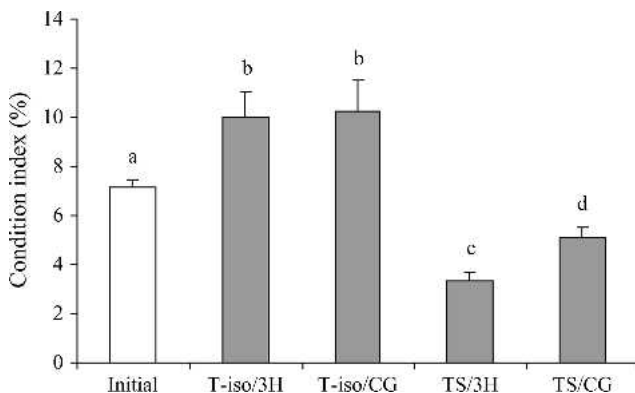


Figure 2. Condition indices for *Clinocardium nuttallii* conditioned at 16°C with different diets over a 13-wk period. Means with different superscripts differ significantly (Student-Newman-Keuls multiple range test,  $P < 0.05$ , for square-root transformed data). Bars represent standard errors ( $n = 6$ ). T-iso: *Isochrysis galbana* (Tahitian strain), 3H: *Thalassiosira pseudonana*, TS: *Tetraselmis suecica*, CG: *Chaetoceros gracilis*.

In contrast, the proportion of mature oocytes started to increase immediately for those oysters containing growing oocytes at the beginning of the experiment (Muranaka & Lannan 1984).

Autumn has often been described as a difficult time for conditioning bivalve broodstock (e.g., Gérard & Robert 1999). Recent studies of Chávez-Villaba et al. (2002) and Fabioux et al. (2005) demonstrated that the spent oysters (*C. gigas*) obtained during this time of the year after major summer spawning events, could not reconstitute their stock of germ cells to initiate gametogenesis when held (conditioned) at elevated temperature and provided with sufficient food, as used with the routine conditioning method. Instead, such spent oysters had to experience a winter season, either under natural or laboratory-accelerated rhythms of variations in temperature and photoperiod (the cold water temperatures of winter, however, appeared to be more involved), before a new reproductive cycle started, and this was characterized by the presence of growing oocytes in the gonads (Chávez-Villaba et al. 2002, Fabioux et al. 2005). The presence of growing oocytes could indicate that the oysters are no longer in a dormancy period (when they are not receptive to signals from the environment) and, as a result, successful artificial conditioning of the oysters can only be achieved with those animals that have developed at least the growing oocytes in their gonads (Chávez-Villaba et al. 2002).

These findings for oysters may have important implications for explaining failure in the conditioning process as experienced in the present study. Gonads of *C. nuttallii* were largely spent at the start of the conditioning experiment. This was because of the fact that the broodstock cockles had spawned twice (one on their arrival at the CSR and the other weeks later after a reconditioning period) before being used in the conditioning experiment. Hence, the cockles may have entered a dormancy

period in which they were not able to become reproductively active without an environmental trigger, such as a change in photoperiod and/or temperature. Interestingly, the cockles that were exposed to a cold-temperature regime of 2.5°C (the BZP treatment) were able to start developing growing oocytes, albeit at a low developmental rate. This shows that a cold-water cycle is important in the initiation of gametogenesis for spent cockles. In the wild, initiation of gametogenesis in most temperate bivalves, including *C. nuttallii*, has been commonly observed in winter when water temperature is low (Gallucci & Gallucci 1982, Darriba et al. 2004, Fabioux et al. 2005, Barber & Blake 2006). Further research is required to investigate the effect of temperature and initial gonadal stage, and therefore timing of broodstock collection, on the conditioning success of *C. nuttallii* in captivity.

Previous studies on molluscs, most of them carried out on abalone, have shown that the development rate of larvae and gonadal maturation are functions of cumulative difference between the holding temperature (below the species' upper thermal limit) and the BZP. The value of this function, known as the effective accumulative temperature (EAT), is constant for each developmental stage and once quantified offers a means of predicting the onset of each developmental stage at particular temperatures (Kikuchi & Uki 1974a, Kikuchi & Uki 1974b, Seki & Kan-no 1977, Grubert & Ritar 2004a, Grubert & Ritar 2004b, Zhao et al. 2004).

Mann (1979) described the relationship between gonadal maturation and temperature using the equation:

$$D = d(t - t_0)$$

where D is the EAT (degree-days, °C-D), d is the number of days required to achieve a ripe state, t is the temperature to which the animals are exposed, and t<sub>0</sub> is the temperature below which no evidence of gonad development is found (the BZP for gonadal development). By knowing the EAT for gonadal maturation of a target species, it is possible to predict the duration of time to condition the broodstock at any given temperature. Mann (1979) found that for the oyster *C. gigas*, D is 592°C-D and t<sub>0</sub> is 10.6°C. Previously at the CSR, we had successfully conditioned one batch of *C. nuttallii* broodstock, collected in early November (initiation of gametogenesis) and maintained at a mean temperature of 14.5°C, to spawn within 58 days after collection. Another batch of broodstock, which was collected in early June (reproductive season) and held at a mean temperature of 16.0°C, spawned again 42–58 days after the first spawning (unpublished data). Based on these observations, it is estimated that the EAT for gonadal maturation of *C. nuttallii* is 550–730°C-D, with the use of the formula of Mann (1979) and assuming a similar BZP between gonadal and larval (2.9°C) development (Hahn 1989, Grubert & Ritar 2004a, Grubert & Ritar 2004b). However, more batches of the broodstock should be examined to confirm the range of EAT for gonad maturation in *C. nuttallii*. Nonetheless, the EAT notion is only a rough indicator for predicting the timing of ripening in

bivalves, because it is dependent on the extent of gonad maturation when conditioning is started, and is also influenced by food availability, diet quality, and the conditioning temperature (Muranaka & Lannan 1984, Utting & Millican 1997).

Gametogenesis in certain bivalve species has been reported to occur at relatively low temperatures, for example between –1.5°C and 5°C, for many temperate pectinid species (Barber & Blake 2006). Indeed, a temperature of 2.5°C was still not fully inhibitory to the gonadal development of *C. nuttallii*. It thus appears that the BZPs for gonadal development in these cases, if determined accordingly, would be much lower than that of about 10°C reported for the oysters *C. virginica* (Loosanoff & Davis 1952) and *C. gigas* (Mann 1979). However, there is still the question of whether the two minimum threshold temperatures for initiation of gametogenesis (proliferation of gonial cells) and for germ cell maturation (vitellogenesis) are the same among different bivalve species or populations (Sastry 1968, Sastry & Blake 1971, Ruiz et al. 1992, Fabioux et al. 2005).

Data on the effect of dietary items on broodstock conditioning of *C. nuttallii*, with respect to egg and larval quality, were not available because of the immature state of the broodstock at the end of the trials. However, the reduced condition indices, obtained when the microalga TS was included in the diets, suggest that TS should be eliminated from use in conditioning *C. nuttallii*, at least with the tested ratio of combinations (with 3H and CG). The nutritive value of TS as a food item for larval and postlarval bivalves, either as a single-algal diet or as a component of mixed-algal diets, has been found to range from poor to high (Laing & Millican 1986, O'Connor et al. 1992, Utting & Spencer 1991, Albentosa et al. 1996). This discrepancy can at least be attributable to differences in the nutritional requirements of bivalves (Robert et al. 2001). Our observations showed that TS cells in cockle feces examined during the experiment were mostly intact, indicating that the digestibility of TS for *C. nuttallii* was poor. A similar result was found during broodstock conditioning of the scallop *Pecten fumatus* (Heasman et al. 1996). The poor digestibility of TS may have resulted from the difficulties encountered in digesting the theca of this microalga (Epifanio 1979, Heasman et al. 1996). However, the small, tan-colored digestive glands of *C. nuttallii* fed both diets with TS otherwise indicate that TS or the tested diets with TS are somewhat agitating to cockle broodstock. The dietary effect on *C. nuttallii* broodstock performance needs re-examination.

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