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Hatchery broodstock conditioning of the blue mussel *Mytilus edulis* (Linnaeus 1758). Part I. Impact of different micro-algae mixtures on broodstock performance

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Abstract Blue mussel (*Mytilus edulis*) broodstock collected from the Irish Sea during wintertime (November) was conditioned with three different microalgae diets. Positive flagellates (PF) treatment consisted of *Pavlova lutherii*, *Isochrysis galbana* (T-Iso), and *Chaetoceros calcitrans* (1:1:1). Positive diatoms (PD) treatment consisted of *Pavlova lutherii, Chaetoceros calcitrans*, and *Skeletonema costatum* (1:1:1). Broodstock animals in the PF and PD treatments were fed a total of 2.4×10^{11} algae cells per day. Animals in the negative flagellates (NF) treatment received only 1/8th of the total amount of algae of the PF diet. The conditioning diets had an impact on spawning success and broodstock fecundity but not on hatching rate, which was similar in all three groups. The best results were obtained with the PD diet where 84% of the conditioned animals spawned and females released 5.0×10^6 eggs on average. Animals belonging to the PF and NF treatments released, on average, only 3.6×10^6 and 1.6×10^6 eggs, respectively. Although the amounts of algae provided to the broodstock animals had no effect on the hatching rate, the D-larvae resulting from the NF treatment were smaller in size than the larvae from the other treatments. Biochemical analysis of the different broodstock groups at the end of the experiment revealed higher carbohydrate levels in group NF than in PF and PD, supporting the theory that gametogenesis is mainly supported by the energy from the glycogen reserves. As far as we are aware this is the first study describing hatchery broodstock conditioning of blue mussels under fully controlled conditions.

Keywords Hatchery · Bivalves · Broodstock · Conditioning · Spawning · Biochemical composition · Blue mussel · *Mytilus edulis*

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Abbreviations

PD	Positive diatoms treatment
PF	Positive flagellates treatment
NF	Negative flagellates treatment
DW	Dry weight
AFDW	Ash free dry weight
OM	Organic matter

Introduction

In recent years interest in producing mussel (*Mytilus edulis*) spat in hatcheries in The Netherlands has increased because the wild spat supply cannot satisfy demand. Especially during the last two decades, some periods of intense fishing followed by frequent failing of recruitment in the Wadden Sea (The Netherlands) has caused an overall decline in wild and production stocks (Herlyn and Millat 2000; Beukema and Dekker 2007). As a result of the decline in available mussel seed, the mussel industry in The Netherlands has been greatly affected (personal comment J.W. Kosten, former auctioneer Dutch mussel auction, Yerseke).

Reproduction of the blue mussel in wild populations follows seasonal changes of environmental factors such as food availability and water temperature (Bayne et al. 1978; Newell et al. 1982; Rodhouse et al. 1984; Pipe 1985). In European waters, development of the gonads begins in autumn and continues through winter, with spawning taking place the following spring and summer (Gabbot and Bayne 1973; Pieters et al. 1980).

For hatcheries to be able to produce mussel spat all year round, good broodstock conditioning is essential. Although it is possible to use a commercial formulated diet for *Mytilus* spat (Nevejan et al. 2007), no such diet is available yet for mussel broodstock conditioning or larval rearing. Because of this, hatcheries still greatly depend on the use of micro-algae (Muller-Fuega 2000; Brown 2002). In a study by Bayne et al. (1978) raw seawater with added diatoms was used for broodstock conditioning but resulted in a low spawning success. Other studies described the laboratory spawning and larvae rearing of several *Mytilus* species (Beaumont et al. 1993, Matson et al. 2003). But even though *M. edulis* is one of three commonly cultured mussel species in China (Zhang 1984) no information is available on the conditioning of mussel broodstock under fully controlled conditions.

Studies showed that increasing the water temperature initiates gametogenesis in bivalves but fecundity is mainly influenced by quality and quantity of the algal diet (Lubet 1976; Utting et al. 1997). Feeding bivalves with a mixture of micro-algae has become common practice in hatcheries since different micro-algae species vary considerable in their nutritional value and only by mixing species can optimal food conditions be obtained (Brown et al. 1989; Brown 2002; Utting et al. 1997).

The primary aim of the experiments described below was to find a broodstock-conditioning diet that maximizes fecundity, egg quality, and larval viability and is suitable for use in commercial hatcheries. In Part I of this study we describe the effects of three conditioning diets consisting of different mixtures of micro-algae at different concentrations on the reproductive output of *M. edulis*. In Part II we describe the usefulness of formulated feeds for broodstock conditioning.

Materials and methods

Experimental setup

Mussels from the Irish Sea were graded with an industrial grader that selected individuals with a shell-width between 1.8 and 2.2 cm and a length of 5.3 cm (±0.37). They were divided randomly into six conditioning groups, weighing exactly 3 kg, of approximately 140 individuals each. Animals were placed at the bottom of 40 l tanks with 1 μm, UV-filtered seawater from the Oosterschelde, a sea basin in the southwest of the Netherlands. A gravity tank kept water flow constant at 1.2 l min^{-1}. Tanks were cleaned weekly with a mixture of dilute acetic and hydrochloric acid. During the first week temperature was kept constant at 8.2°C (±0.9°C). In the second week the temperature was raised gradually to a final 18°C. The experiment lasted 46 days and there were six different treatments. Part I compares all algae-based treatments, while the other treatments are compared in Part II of this study.

Those in the positive flagellate (PF) group received a mixture of *Pavlova lutherii* (CCAP 931/1), *Isochrysis* sp. (T-Iso, CCAP 927/14), and *Chaetoceros calcitrans* (CCY 0387) (1:1:1, based on cell counts). Those in the positive diatom (PD) group received *Pavlova lutherii*, *Chaetoceros calcitrans*, and *Skeletonema costatum* (CCAP 1077/5) (1:1:1, based on cell counts). Mussels in the PF and PD groups were fed a total amount of 2.4×10^{11} algae cells a day. This was achieved by adding a constant concentration of algae of 158,000 cells ml^{-1} to the conditioning tanks. The third group, the negative flagellates (NF) group, only received 1/8th of the total amount of algae of the PF diet. The daily amount of algae (supplemented with 1 μm, UV-filtered seawater to obtain a volume of 50 l) was given to the mussels 7 days a week at a constant rate of 37 ml min^{-1}, over a period of 22 h, by using a peristaltic pump. After cleaning of the tanks, 158,000 algae cells were added as background.

Micro-algae were batch cultured in 100-l plastic bags. The filtered (1 μm), UV-treated seawater was chlorinated (0.1 ml l^{-1}) for 24 h, neutralized with 0.1 g l^{-1} thiosulfate and enriched with Walne medium and CO_2 before inoculation. The pH of the cultures was kept between 7 and 8 by adjusting the amount of CO_2. Algae were grown under 24-h light conditions, at a temperature of 21 ± 1°C, and harvested daily in the exponential growth phase. Algae concentrations were counted daily by using a Bürker counting cell.

The state of gonad development was checked at the start of the experiment by microscopic observation of thin sections of the gonads; this revealed that no eggs or sperm were present in the mantle.

Spawning and larval rearing

After 6 weeks conditioning, 100 individuals of each group were cleaned with filtered seawater and placed in spawning tanks. The remaining animals were placed in running seawater for about 2 h to depurate and subsequently frozen at −18°C for biochemical analysis.

The base of the spawning tank was covered with a black plastic sheet to provide a dark background against which released gametes were easily seen. Spawning was initiated by providing a thermal shock. Therefore temperature was raised rapidly from 18°C to 30°C at which it was kept for 20 min. Spawning females were immediately removed from the spawning tank and individually placed in a 300-ml beaker, with filtered seawater at 18°C.

Males were also removed and placed in a 40-l tank with running seawater at 18°C. For each spawning individual the time between applying the thermal shock and spawning was recorded. Mussels that were unable to spawn were sacrificed and histologically checked for sex and state of gametogenesis by taking a thin slice from the mantle and investigating it under a light microscope.

Spawning females belonging to one treatment were divided into three sub-groups. The number of eggs released by each spawning female was counted by suspending them in 1 l of filtered seawater and counting a 50-µl sample. Subsequently, egg samples were taken for analysis. Eggs were fertilized by submersing them for 20 s in a tank with spawning males from the corresponding group. Embryos of each subgroup were stocked in 100-l tanks at a concentration of 15 embryos ml^{-1}. To each tank 2.4×10^9 cells of *P. lutherii* were added to provide food for early D-larvae. After 48 h D-larvae were counted, measured and checked for deformation.

Biochemical analysis

Proximate analysis was carried out on the broodstock animals at the start (November 2006) and at the end (January 2007) of the experiment. The animals were kept at −18°C before analysis. The meat of 17–28 animals was dried at 63°C for 5 days to determine dry weight (DW). Crude ash was determined by weighing the residue remaining after heating at 550°C for 5 h in a muffle oven. Organic matter (OM) was calculated as the difference between DW and ash content. Crude protein was determined on the basis of the nitrogen (N) content, determined according to the Kjeldahl method (EG 1993). Crude fat was determined after hydrolysis (EG 1998). The carbohydrate level was estimated by determining the starch content polarimetrically (EG 1999).

To determine the algal DW, the algae concentration was counted six times by using a Bűrker counting chamber after which 40 ml of the algae mixture was filtered on pre-combusted and pre-weighed GFC filters. The filters were subsequently dried at 63°C for 48 h. After weighing they were combusted at 450°C for 5 h to determine the ash weight.

Hatching rate was defined as the percentage of larvae recovered 2 days after fertilization and was calculated for the three sub-groups per treatment. Larvae of the three sub-groups were combined in one sample per treatment for length (dorsal–ventral) measurements. A reflection of the larval population size per treatment was aimed at by measuring very large samples (>1,000 larvae). Samples were preserved in a lugol solution and measured with Clemex Vision® software.

Data analysis

The homogeneity of variances of means was tested by use of the univariate test (Levene's test). Significant differences were detected by using one-way ANOVA, P being set at 0.05. The Tukey HSD test was used for post-hoc comparison.

Results

Algal DW and AFDW

The average DW and AFDW of the algal mixtures PF and PD were very similar (Table 1). Based on these data it was decided to base the algae diets on cell counts instead of DW.

Table 1 Dry weight (DW) and ash-free dry weight (AFDW) of the two algal mixtures PF and PD (average ± SD)

($n = 6$)	PF	PD
DW (pg/cell)	22.6 (±1.8)	21.7 (±1.6)
AFDW (pg/cell)	19.3 (±0.6)	18.6 (±0.9)

PF = positive flagellate group, *Pavlova lutherii*:*Isochrysis galbana* (T-Iso):*Chaetoceros calcitrans* (1:1:1), 2.4×10^{11} cells

PD = positive diatom group, *Pavlova lutherii*:*Chaetoceros calcitrans*:*Skeletonema costatum* (1:1:1), 2.4×10^{11} cells

Spawning success, fecundity and hatching rate

After 6 weeks conditioning, survival of broodstock was 89% (PD), 84% (PF), and 82% (NF). After applying a temperature shock to initiate spawning, reaction time of broodstock receiving the PF and PD diet was less than 45 min while reaction time of the NF group was 3.5 h.

The amount of spawning success varied between the three conditioning groups. The best results were obtained with the PD and PF diets with 80% of the conditioned animals spawning while only 17% of the NF group could be triggered to release gametes (Fig. 1).

Fecundity also differed between the three conditioning groups. Mussels that received the PD diet showed the highest fecundity with 51% of spawning females releasing 5×10^6 eggs or more. On the other hand only 22% of the PF females released 5×10^6 eggs or more. None of the females of the NF treatment released $>5 \times 10^6$ eggs. The average numbers of eggs released by females was 5×10^6 (PD), 3.6×10^6 (PF), and 1.7×10^6 (NF) (Fig. 2).

Average hatching rate was 71%. There was no significant difference in hatching rate between the different conditioning groups (Table 2).

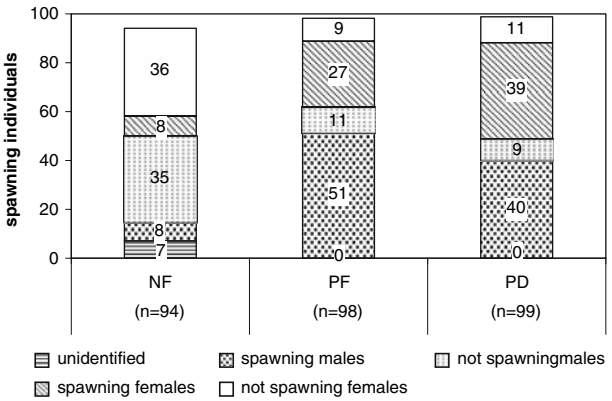

Fig. 1 Number of spawning individuals resulting from conditioning with different diets. NF = negative flagellate group, *Pavlova lutherii*:*Isochrysis galbana* (T-Iso):*Chaetoceros calcitrans* (1:1:1), 3.0×10^{10} cells; PF = positive flagellate group, *Pavlova lutherii*:*Isochrysis galbana* (T-Iso):*Chaetoceros calcitrans* (1:1:1), 2.4×10^{11} cells; PD = positive diatom group, *Pavlova lutherii*:*Chaetoceros calcitrans*:*Skeletonema costatum* (1:1:1), 2.4×10^{11} cells

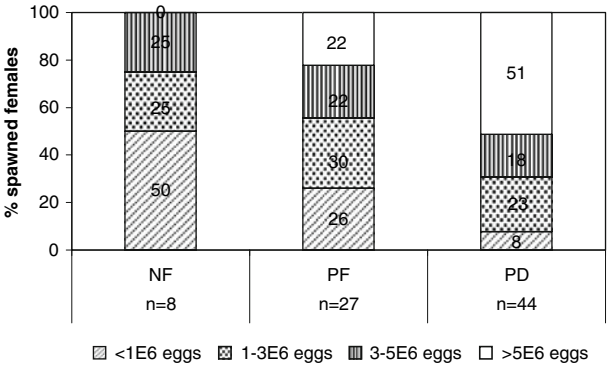

Fig. 2 Percentage of spawned females per category of amount of eggs released. NF = negative flagellate group, *Pavlova lutherii*:*Isochrysis galbana* (T-Iso):*Chaetoceros calcitrans* (1:1:1), 3.0×10^{10} cells; PF = positive flagellate group, *Pavlova lutherii*:*Isochrysis galbana* (T-Iso):*Chaetoceros calcitrans* (1:1:1), 2.4×10^{11} cells; PD = positive diatom group, *Pavlova lutherii*:*Chaetoceros calcitrans*:*Skeletonema costatum* (1:1:1), 2.4×10^{11} cells

Table 2 Hatching rate (%) resulting from conditioning with the three different conditioning diets

($n = 3$)	Hatching rate (%) (average ± SD)
NF	71.0 (±4.9)
PF	71.9 (±4.4)
PD	71.1 (±9.1)

NF = negative flagellate group, *Pavlova lutherii*:*Isochrysis galbana* (T-Iso):*Chaetoceros calcitrans* (1:1:1), 3.0×10^{10} cells

PF = positive flagellate group, *Pavlova lutherii*:*Isochrysis galbana* (T-Iso):*Chaetoceros calcitrans* (1:1:1), 2.4×10^{11} cells

PD = positive diatom group, *Pavlova lutherii*:*Chaetoceros calcitrans*:*Skeletonema costatum* (1:1:1), 2.4×10^{11} cells

D-larvae size and shape

The size of D-larvae was measured 48 h after fertilization. From each treatment a minimum of 1,020 larvae was measured. The D-larvae of group NF were smaller than those of groups PF and PD. A total of 33% of the larvae belonged to size class 80–90 μm and only 10% belonged to size class 100–110 μm. The larvae of the PF and PD groups were similar in size with 34% belonging to size class 100–110 μm and only 1–3% belonging to size class 80–90 μm (Fig. 3).

In none of the three conditioning groups were anomalies observed in the shape of the D-larvae.

Biochemical composition

The mean dry weight per individual mussel was lowest for the animals that received the least amount of algae, i.e. the NF treatment. These animals lost weight during the conditioning period since their individual weight went down by 23.7% from 2.23 g to 1.7 g

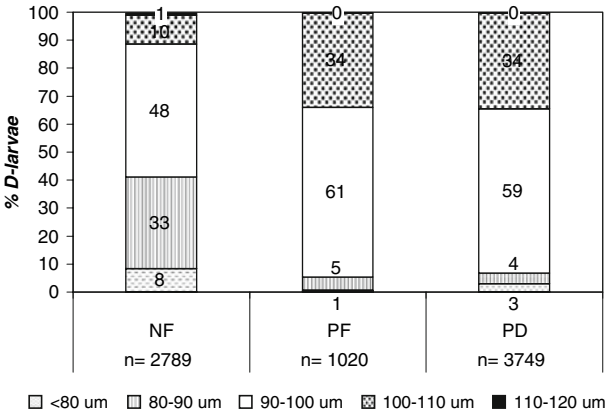

Fig. 3 Percentage of different sizes of D-larvae resulting from the three broodstock diets. NF = negative flagellate group, *Pavlova lutherii*:*Isochrysis galbana* (T-Iso):*Chaetoceros calcitrans* (1:1:1), 3.0×10^{10} cells; PF = positive flagellate group, *Pavlova lutherii*:*Isochrysis galbana* (T-Iso):*Chaetoceros calcitrans* (1:1:1), 2.4×10^{11} cells; PD = positive diatom group, *Pavlova lutherii*:*Chaetoceros calcitrans*:*Skeletonema costatum* (1:1:1), 2.4×10^{11} cells

Table 3 Chemical composition of broodstock animals before (initial) and after (NF, PF, PD) conditioning with the three different diets

	Initial	NF	PF	PD
Sample size	25	27	25	18
Proteins (%)[a]	53.8	55.6	60.0	61.0
Carbohydrates (%)[a]	20.0	16.8	13.8	12.7
Lipids (%)[a]	6.7	8.2	8.3	7.7
OM (%)[a]	89.1	87.0	87.5	89.6
Average DW/individual (g)	2.23	1.70	2.04	2.35
Average OM/individual (g)	1.98	1.48	1.79	2.10

[a] Percentages on DW

Initial = before conditioning

NF = negative flagellate group, *Pavlova lutherii*:*Isochrysis galbana* (T-Iso):*Chaetoceros calcitrans* (1:1:1), 3.0×10^{10} cells

PF = positive flagellate group, *Pavlova lutherii*:*Isochrysis galbana* (T-Iso):*Chaetoceros calcitrans* (1:1:1), 2.4×10^{11} cells

PD = positive diatom group, *Pavlova lutherii*:*Chaetoceros calcitrans*:*Skeletonema costatum* (1:1:1), 2.4×10^{11} cells

(Table 3). There was no difference in ash content, leading to an organic matter content of 87–89% for all treatments.

Proximate analysis of the animals revealed no big difference in lipid content between the treatments, while the protein content tended to be higher for the animals receiving the high amounts of algae, i.e. PF and PD (Table 3). The broodstock animals of the NF treatment on the other hand seem to have a higher carbohydrate content of 16.8% DW which is 21% and 32% higher than for the animals belonging to the PF and PD treatments, respectively (Table 3).

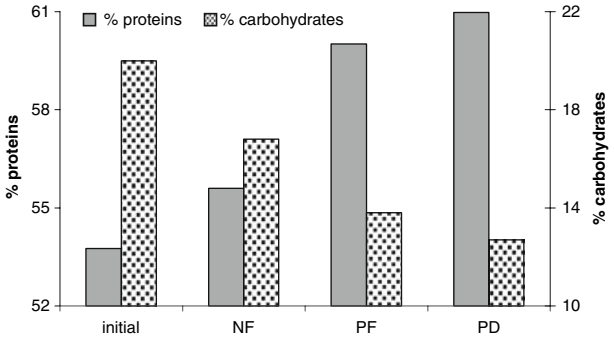

Fig. 4 Relationship between protein and carbohydrate content in broodstock animals before (initial) and after conditioning with the three different diets. NF = negative flagellate group, *Pavlova lutherii*:*Isochrysis galbana* (T-Iso):*Chaetoceros calcitrans* (1:1:1), 3.0×10^{10} cells; PF = positive flagellate group, *Pavlova lutherii*:*Isochrysis galbana* (T-Iso):*Chaetoceros calcitrans* (1:1:1), 2.4×10^{11} cells; PD = positive diatom group, *Pavlova lutherii*:*Chaetoceros calcitrans*:*Skeletonema costatum* (1:1:1), 2.4×10^{11} cells

Figure 4 shows the relationship between proteins and carbohydrates in whole broodstock animals before and after conditioning.

Discussion

Environmental stress reduces the spawning success and fecundity of mussels in proportion to the decline of energy available for gamete production (Bayne et al. 1978). In this study spawning success and fecundity varied between the three broodstock groups as a result of the differences in the diets they received. The NF diet clearly did not provide enough energy for adequate spawning success nor fecundity with only 18% of the females spawning, releasing an average of merely 1.6×10^6 eggs per female. Animals that received the PF and PD diets had a much higher spawning success with 75% and 78%, respectively, of the females spawning. Minimum and maximum fecundity for a female of 980 mg dry flesh weight, derived from 3 years of observation of *M. edulis* from Petpeswick Inlet, Nova Scotia, was calculated to be 5.5×10^6 and 10.2×10^6 eggs (Thompson 1979). Although the DW of 2.35 g per individual of the broodstock animals in this study is much higher, the maximum amount of eggs produced by one female was 11.2×10^6 (PD diet). Females of the PD group had the highest average fecundity of 5.0×10^6 eggs (compared to an average of 3.6×10^6 eggs spawned by females that received the PF diet) indicating that this diet was better for providing energy for gamete production. Since the DW of the two algae mixtures PD and PF was very similar (Table 1), this different fecundity can be attributed to the difference in algae species. Besides the gross composition of algae, many other factors contribute to their nutritional value, for example vitamins, minerals, pigments, and sterols (Brown et al. 1989; Brown 2002). In comparison to the flagellates, the diatoms used in this experiment are richer in cholesterol, which might have contributed to the better conditioning properties of the PD diet in comparison to the PF diet (Patterson 1992).

Studies showed that eggs produced under nutritional stress are smaller and contain less organic matter than eggs produced under more optimal conditions (Bayne et al. 1978). Unfortunately, in this study egg size was not measured. But the D-larvae of the NF group were smaller in size than D-larvae produced by the PF and PD groups (Fig. 3).

Hatching rate is mainly determined by endogenous reserves laid down in the eggs during vitellogenesis (Utting et al. 1997). For example, the hatching rate of *Crassostrea gigas* eggs is affected by the biochemical composition of the diet fed to the adults (Uriarte et al. 2004). This study shows that the hatching rate of *M. edulis* does not seem to be affected by composition or amount of algae diet available to the adults during conditioning.

In mussels, the early stages of larval development (up to the prodissoconch I-stage) represent a period of intense morphogenetic activity during which the larvae totally depend on their stored energy reserves. During this period, the effect of stress experienced by the adults during conditioning will have its maximum impact on the larval development (Bayne 1972). In this study no increase in abnormal D-larvae development for the NF group was observed, although this group experienced nutritional stress during conditioning, illustrated by the longer spawning response in comparison to the other two conditioning groups. This is in contrast with the findings of Bayne (1972), who showed that nutritional stress in the adult leads to an increase of abnormal D-larvae development. However, in his experiment animals coming straight from the field were compared with animals conditioned in the laboratory. Furthermore, adults were induced to spawn by increasing the temperature by 5°C and injection of 1 ml 0.5 mol l^{-1} KCl into the adductor mussel. By using this more extreme method to induce spawning it is possible to trigger the release of gametes from animals that were not properly conditioned which in turn can result in eggs with reduced viability (Utting et al. 1997).

In European waters, gametogenesis is usually initiated in October or November and continues throughout the winter. Vitellogenesis normally takes place from November to January after which there may be a "resting" period until spring when temperatures start to rise and gonad development continues (Bayne et al. 1975). During gametogenesis carbohydrate (free sugar) reserves are utilized for gonad development (Gabbot and Bayne 1973; Zandee et al. 1980). This is in accordance with current study where we find lower levels of carbohydrate with increased fecundity. This is also illustrated by the fact that the PF and PD groups had 18% and 24% less carbohydrate, respectively, than the NF group, due to their higher fecundity.

Proteins serve as the main energy source for mussels during late winter and early spring. During gametogenesis protein content in the mantle decreases but total amount of proteins in the whole animal can still increase since most of it is stored in non-mantle tissues (Gabbot and Bayne 1973; Bayne et al. 1975). In this study we also see a slight increase of protein levels in all treatments, even in the NF group which received only 0.3×10^{11} algae cells a day, which equals 0.203% of the dry meat weight of the mussels at the beginning of the conditioning period.

During summer lipids are the main source of energy production in growing mussels but from autumn until spring lipid reserves that were build up during summer are used only for gametogenesis, whereas proteins are used for both energy production and gametogenesis (Zandee et al. 1980). This is in accordance with current study where we see no significant difference between total lipid content of the PF and PD groups and of the initial group since during gametogenesis lipids are merely relocated from the mantle into the gametes instead of being used for energy production.

Conditioning conditions such as temperature and quality and quantity of food required to obtain maximum fecundity, egg quality, and larvae viability inevitably differ according to the time of year animals are taken from the field and their gametogenetic state. A food ration advised for conditioning most bivalve species is 3–6% of the dry meat weight in dry weight of algae per day (Utting 1997). This study shows, however, that under given experimental conditions a daily algae ratio of 1.6% is sufficient to obtain ample fecundity,

egg quality, and larval viability. Furthermore, a diet consisting mainly of diatoms gives better results than a diet dominated by flagellates.

This study demonstrates that out-of-season conditioning of mussel broodstock is feasible. This offers the possibility for commercial hatcheries to produce blue mussel seed all year round to optimize their investments.

Recommendations

Cultivation of microalgae is one of the major costs of a bivalve hatchery. Given the large difference between studies of daily algae rations needed to condition mussel broodstock (1.6–3.6% of the dry meat weight in dry weight of algae per day), additional studies are necessary to further optimize the conditioning process, especially when starting with lean adults.

This study showed that low food availability during conditioning does not cause abnormal D-larvae development. But on many occasions where mussels were conditioned under natural conditions, abnormal prodissoconch I larvae development was substantial, sometimes reaching levels as high as 80%, depending on the source of the adults (observations made by the authors). This has led to the hypothesis that abnormality in D-larvae development is caused by factors other than nutritional, for example pollution. Since the occurrence of large quantities of deformed larvae can reduce natural spat fall significantly, the cause of abnormal larvae development urgently warrants further study.

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