水研センター研報, 第3号, 1−6, 平成14年
Bull. Fish. Res. Agen., No. 3, 1−6, 2002

Brown nodules formed in the appendages of *Penaeus monodon* postlarvae cultured in a hatchery in Madagascar

Motohiko SANO[*1], Hideto IWAKAWA[*2], Masanori SUEMITSU[*2], Megumi MINAGAWA[*1,4]
Juliette RASOARINIRO[*3], and Louisette RZAFINDRADOANY[*3]

Abstract Brown nodules, leading to shrimp mortality, frequently formed in the appendages of *Penaeus monodon* postlarvae cultured in a hatchery in Madagascar. The nodules resulted from a melanized hemocytic host reaction, which was activated by the necrosis of the cuticular epithelium of the appendages. In histopathological examinations, no evidence of bacterial invasion was detected in the postlarvae with brown nodules. The nodules were frequently formed in postlarvae that were starved for four days. The brown nodules in appendages disappeared after the postlarvae were transferred to a clean and less-densely reared aquarium. Furthermore, addition of formalin at 25ppm to the rearing water inhibited the formation of brown nodules, although the mechanism was not clear. These results suggest that the brown nodule formations in the appendages of the hatchery-reared postlarvae of *P. monodon* were probably caused by a combination of several factors related to water quality and feeding and not by bacterial infection.

Key word: *Penaeus monodon*, brown nodule formation, appendages necrosis

The Japan International Cooperation Agency and the Madagascar Government have conducted the project, Aquaculture Development Project in the Northwest Coastal Region, since 1998 to promote small-scale aquaculture of *P. monodon* among the fishermen in the Northwest Coastal Region. In this project, the transfer of technology has included the shrimp seed production, shrimp growout systems and pond management at the Centre de Developpement de la Culture de Crevettes (Center for the Development of Shrimp Culture : CDCC), which has two facilities, a hatchery and growing-out ponds in Mahajanga (**Fig. 1**). The hatchery produced more than 10 million postlarvae of *P. monodon* in the 2000 fiscal year.

Brown nodule formation in the postlarvae appendages, such as the pereopods, pleopods, and antennae, has been present since the beginning of the CDCC hatchery seed production in 1998. The for-

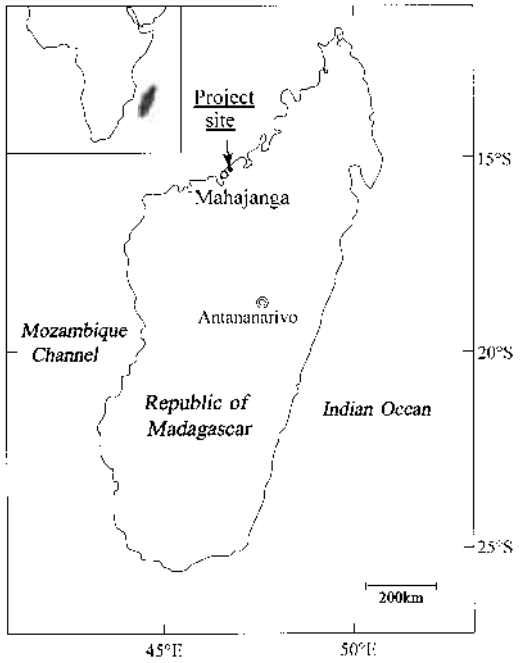

Fig. 1. Location of the project site in Mahajanga, Madagascar.

2002年3月13日受理（Accepted on March 13, 2002）
水産総合研究センター業績 A 第12号（Contribution No.A 12 from Fisheries Research Agency）
[*1]西海区水産研究所石垣支所 〒907-0451 沖縄県石垣市字浮海大田148-446（Ishigaki Tropical Station, Seikai National Fisheries Research Institute, Ishigaki, Okinawa 907-0451, Japan）
[*2]国際協力事業団 〒151-8558 東京都渋谷区代々木2-1-1（Japan International Cooperation Agency, Yoyogi, Tokyo 151-8558, Japan）
[*3]マダガスカルエビ養殖センター（Centre de Developpement de la Culture de Crevettes, Mahajanga B, P. 85, Madagascar）
[*4]現住所 西海区水産研究所企画連絡室 〒850-0951 長崎県長崎市国分町3-30（Research Planning and Coordination Division, Seikai National Fisheries Research Institute, Nagasaki 850-0951, Japan）

mation ultimately leads to shrimp mortality. The symptoms were found in the postlarvae reared in 10-ton tanks with slightly dirty rearing water in which some protozoa were observed. The postlarvae with brown nodules were not actively swimming and usually had empty guts, meaning that they consumed very little. The CDCC seed production operation usually took place from April to January. Brown nodule formation was more frequently observed from November through January, when the water temperatures were higher, ranging from 28 to 30℃, than it was from July through September, when the water temperature ranged from 26 to 28℃. In practice, the addition of formalin to the rearing water tanks seemed to decrease the incidence of brown nodules in the cultured shrimp.

Brown nodule formation in the appendages resembles the symptoms of vibriosis, such as brown spot disease and appendage necrosis, which have been previously reported (Lightner, 1988 ; Lightner, 1996). It had been assumed that the brown nodule formation was caused by vibriosis. However, a causative bacterium was not microscopically observed in wet-mount specimens, and our preliminary bacterial isolation trial from the shrimp with brown nodules using marine agar and TCBS agar showed that there were no dominant colonies specifically isolated from the shrimp. The cause of the formation of brown nodules in the appendages of *P. monodon* cultured in CDCC remained unclear.

The first author (M. S.) had the opportunities to join this project in Madagascar in 1999 and 2000 as an expert in the field of shrimp pathology and to investigate this symptom. In this report, we conducted a histopathological examination of the postlarvae and studied the effects of starvation on the postlarvae in order to determine the cause of brown nodule formation in the appendages of hatchery-reared *P. monodon* postlarvae. To control the formation of the nodules, we examined the effects of adding formalin to the rearing water and transferring the affected shrimp to other clean tanks.

Materials and Methods

Histopathological observations of brown nodules in postlarvae

The postlarvae bearing brown nodules formed in the appendages were collected from 10-ton rearing tanks at the CDCC and fixed in Davidson's fixative solution (Bell and Lightner, 1988). These samples were processed for paraffin sections followed by H&E stain.

Induction of brown nodules in postlarvae by starvation

Eight-day old postlarvae (PL8) with no brown nodules were collected from a 10-ton rearing tank, and 25 individuals were transferred to two 30-liter aquariums with fresh seawater. One aquarium was prepared for the control group, which was fed 0.01 g of a commercial diet (No. 1, Maruha Corp.) twice a day, and the other aquarium was used for the starvation group, which received no feed. One half of the rearing water was exchanged with fresh seawater once a day. After they were reared for four days at 30℃, all shrimp were collected and microscopically examined for brown nodules.

Inhibitory effect of formalin on the occurrence of brown nodules in postlarvae

Twenty-five postlarvae of PL8 with no brown nodules were placed in two 30-liter aquariums. In one group, formalin was added to the aquarium rearing water at a concentration of 25ppm, and the other aquarium, used as a control group, was not supplemented. One half of the rearing water was exchanged with fresh seawater once a day, and formalin was added to one aquarium to maintain the level of concentration in the water. After both groups were starved for four days at 30℃, all shrimp were collected and examined for brown nodules.

Effect of transferring the affected shrimp to a less-densely populated aquarium

Two hundred postlarvae (PL8) with brown nodules were collected from a 10-ton rearing tank and divided into two groups. Each group was transferred to a 30-liter experimental aquarium with a rearing density that was 10-fold lower than that of the 10-ton tank. They were reared for three days at 31℃, receiving 0.04 g of a commercial feed (No. 1, Maruha Corp.) twice a day. One half of the rear-

Fig. 2. *P. monodon* postlarva (PL5) cultured in CDCC showing brown nodule formation in the appendages. Small arrowhead and large arrowhead shows light-brownish nodule at the early stage and clear brown nodule, respectively. Arrows indicate loss of the apical part of the appendage. Note lack of guts contents.

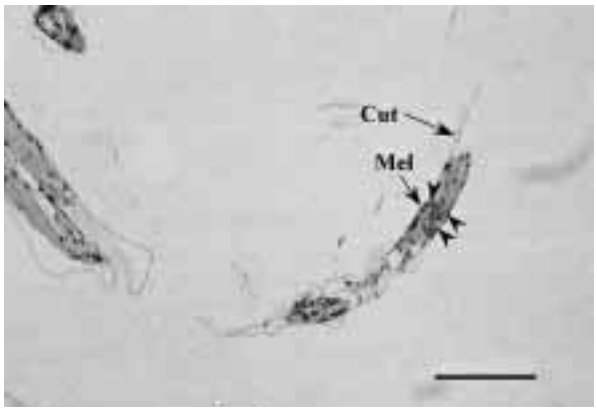

Fig. 3. Histological section of a brown nodule at the early stage in the *P. monodon* postlarva (PL9). The brown nodule at the tip of the 3rd pleopod showed a slightly melanized, hemocytic host reaction. Necrosis of the cuticular epithelium cells were seen (arrowhead). Cut : cuticle layer, Mel : melanization. H&E stain. Bar : 100㎛.

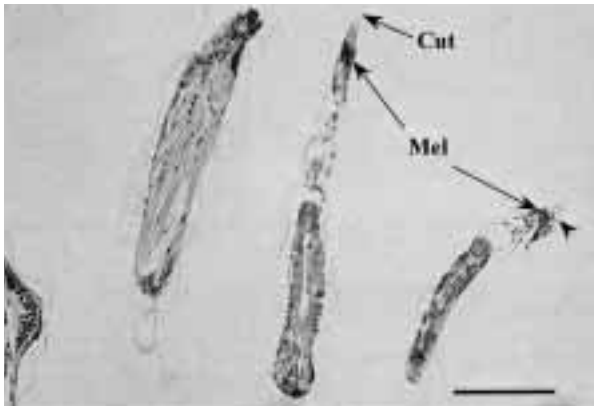

Fig. 4. Histological section of brown nodules in the *P. monodon* postlarva (PL9). Melanized, hemocytic nodules (Mel) were formed at the tip of the 4th and 5th pleopods, and the tip of the 5th pleopod was lost(arrowhead). Cut : cuticle layer. H&E stain. Bar : 100㎛.

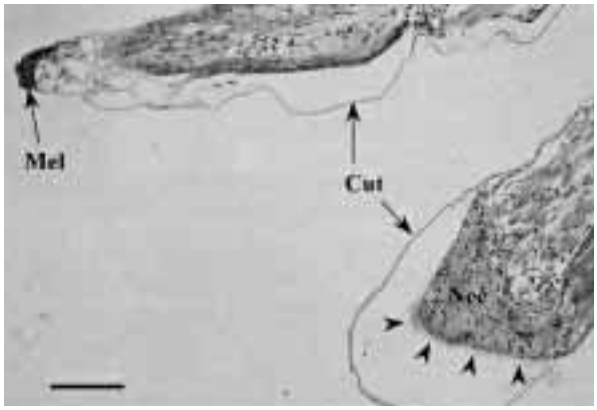

Fig. 5. Histological section of the pleopod infected with bacteria in the *P. monodon* postlarva (PL31) reared in a 10-ton tank in which mass mortality occurred. Note bacterial invasion (arrowhead) in the 3rd pleopod with necrotic muscle tissue (Nec) after rotting of a part of the brown nodule. The brown nodule (Mel) in the 2nd pleopod still remained. Cut : cuticle layer. H&E stain. Bar : 100㎛.

ing water was exchanged with fresh seawater once a day. Five individuals were collected daily from the 30-liter aquarium and from the 10-ton tank and examined for the formation of brown nodules in their appendages.

Results

Gross signs

In the 10-ton rearing tanks, the affected shrimp usually had empty guts. At the early stage, the tip of the appendage was seen as light brown, and then the brown nodule was clearly visible. The loss of the apical part of the appendage subsequently occurred (**Fig. 2**).

Histopathological observation of the brown nodules in postlarvae

The brown nodules found in the tips of appendages were melanized hemocytic host reaction. In the early stage, necrosis of the cuticular epithelium layer at the apical part of the appendage occurred, and a slightly melanized hemocytic nodule (**Fig. 3**) formed in the tip. Melanogenesis then progressed. Bacterial multiply and parasites were not observed in the tips of the appendages at this stage of the development of the nodule. Subsequently, the tips of the appendages were lost (**Fig. 4**). After a part of the brown nodule rotted, bacterial

Table 1. Occurrence of brown nodules in the appendages of *P. monodon* postlarvae (PL8) by starvation

Experimental group	Positive rate (%)* (No. of positive/No. of survival)	Survival rate (%) (No. of survival/No. of shrimp used)
Starvation	86.4 (19/22)	73.3 (22/30)
Control (feeding)	25.0 (7/28)	93.3 (28/30)

*: Significant difference between 2 groups (χ^2test, $P<0.001$).

Table 2. Inhibitory effect of formalin on the occurrence of brown nodules in the appendages of *P. monodon* postlarvae (PL8) caused by starvation

Experimental group	Positive rate (%)* (No. of positive/No. of survival)	Survival rate (%) (No. of survival/No. of shrimp used)
Formalin-treated	20.0 (5/25)	83.3 (25/30)
Control	80.8 (21/26)	86.7 (26/30)

*: Significant difference between 2 groups (χ^2test, $P<0.001$).

Table 3. Positive rate of brown nodules in the appendages of *P. monodon* postlarvae (PL8) after transfer to a less-densely populated aquarium

		Positive rate of brown nodules (No. of positive shrimp/No. of shrimp examined)				
		Days after transfer				
Aquarium No.	No. of shrimp	0	1	2	3	Survival rate (%)
1	100	100(5/5)	60(3/5)	40(2/5)	0(0/5)	90.6
2	100	100(5/5)	80(4/5)	40(2/5)	20(1/5)	71.8
10-ton tank	310000	60(3/5)	80(4/5)	60(3/5)	60(3/5)	71.0

invasion of the appendages occurred easily, and diffuse necrosis of the muscle tissue followed (**Fig. 5**), leading to shrimp mortality.

Induction of brown nodules in postlarvae by starvation

In four days, brown nodules occurred in appendages at a rate of 86.4% among the group that was starved and at a rate of 25.0% among the control (**Table 1**), which was a significant difference between the groups with $P<0.001$ (χ^2test). The nodules were severe in several appendages in the group that was starved, whereas the control group was only slightly affected. A microscopic examination revealed that the nodules formed in the shrimp from the group that was starved were the same as those in the shrimp that were cultured in the 10-ton rearing tanks. The survival rate was 73.3% and 93.3% in the starved and control groups, respectively. Cannibalism contributed to the low survival rate among the starved group.

Inhibitory effect of formalin on brown nodules in postlarvae

Brown nodules in the appendages occurred at rates of 20.0% and 80.8% in the formalin-treated and-untreated groups, respectively, after four days of starvation (**Table 2**). The survival rates were 83.3% and 86.7% in each respective group.

Effect of transferring the affected shrimp to a less-densely populated aquarium

The incidence of brown nodules in the appendages decreased daily in the two experimental groups and was 0% and 20% three days after being transferred (**Table 3**). The survival rates of the experimental groups were 71.8% and 90.6%. In contrast, the positive rate of the nodules in the

10-ton control tank did not change remarkably during the experimental period, and the incidence was high, ranging from 60% to 80%.

Discussion

Histopathological examinations revealed that the brown nodules formed in the appendages of the postlarvae of *P. monodon* that were cultured in the CDCC were a melanized hemocytic host reaction. The hemocyte inflammation and subsequent melanogenesis, which is known as a self-defense mechanism in crustacea (Wago, 1986), were probably induced by the necrosis of the epithelium in the apical part of the appendage. The histopathological examinations showed no evidence of bacterial and parasitic invasions in the postlarvae with the brown nodules in the appendages, which was the same as the results from the wet-mount examination and preliminary bacterial-isolation trial. Furthermore, starvation induced the occurrence of nodules in the postlarvae. This symptom can be controlled by transferring the shrimp to a clean, less-reared tank. These results suggest that the formation of brown nodules in the appendages of the hatchery-reared postlarvae of *P. monodon* is probably caused by a combination of several factors related to water quality and feeding and not by bacterial infection.

A limited incidence of brown nodules in the appendages occurred in the experimental shrimp of the control group which received feed. The cause of the occurrence was not clear, but we assume that there were underlying factor(s) causing the nodules in the shrimp cultured in the CDCC. The CDCC hatchery is located near an estuary of the Betsiboka River, which might influence the quality of the CDCC supplying water, especially from November to January, which is the beginning of the rainy season and higher temperatures. Salinity of supplying water was around 30 from November to January, but it was around 35 from July to September during dry season with lower temperatures. The occurrence of brown nodules in the 10-ton rearing tanks at the CDCC was more frequent from November to January than it was from July to September. Since *P. monodon* is comparatively tolerant to changes in temperature and salinity (Lester and Pante, 1992), it is unlikely that changes in temperature and salinity prompted the nodule to occur. Flegel *et al*.(1992) pointed out that penaeid shrimp larvae are quite sensitive to environmental conditions and toxic substances. We assume that the water used for rearing at the CDCC was influenced by inland water and might have been an underlying factor in the formation of the nodules.

Histopathological examinations showed that the brown nodules lead to rot of the tip of the appendages and subsequently of a part of brown nodules, which results in the invasion of bacteria such as vibrios. Therefore, the formation of brown nodules is a primary cause of vibriosis in the hatchery. There have been many reports in which the causative *Vibrio* species were isolated from moribund or dead penaeid shrimp larvae and postlarvae in hatcheries, and special attention should be given to luminous species, such as *V. harveyi* and *V. splendidus* (Lavilla-Pitogo *et al*., 1990 ; Karunasagar *et al*., 1998 ; Lapvilla-Pitogo and de la Pena, 1998). However, *Vibrio* species are generally thought to be an opportunistic pathogen and secondary cause of shrimp mortality in hatcheries, because these bacteria are a part of the normal bacterial flora in hatcheries and there is no correlation between larval survival and viable cell counts of *Vibrio* in the rearing water (Flegel *et al*., 1992). Since melanogenesis occurs by the infection of *Vibrio* in organs of the penaeid shrimp, such as the gill, lymphoid organs, and appendages (Egusa *et al*., 1988 ; Lightner, 1996), vibriosis are likely to be cited as the cause of mortality when brown spots are observed in the appendages of dead or moribund shrimp larvae and postlarvae. It is possible that brown nodules in the appendages of penaeid shrimp, which are similar to those reported in this study, occur at other hatcheries and are an underlying cause of vibriosis.

Formalin at 25ppm has been used as a practical treatment for brown nodules in shrimp reared in 10-ton tanks at the CDCC. The result lends support to the inhibitory effect of formalin on the formation of brown nodules, but the mechanism is not clear. Further study is needed to determine the mechanism of the inhibitory action of formalin on the formation of brown nodules in *P. monodon* postlarvae.

The affected postlarvae with brown nodules can be recovered after they were transferred to a clean tank with a less-dense population. Although many antibacterial compounds are used in shrimp hatcheries (Baticados *et al*., 1990), the management of water quality is more important for controlling the occurrence of brown nodules than the administration of antibacterial drugs and chemicals, such as formalin.

Acknowledgments

The authors are grateful to Dr. Takeshi Hara of the Japan Fisheries Resource Conservation Association, who is the Management Consultation Team leader of The Aquaculture Development Project in the Northwest Coastal Region in Madagascar, for his encouragement and helpful comments. The first author (M. S.) wishes to thank the Ministere de la Peche et des Ressources Halieutiques du Madagascar, the Fisheries Agency of Japan, the Seikai National Fisheries Research Institute, the Japan International Cooperation Agency, and the Management Consultation Team of the project for the opportunity to conduct on-site research in Madagascar.

References

Baticados, M.C.L., Lavilla-Pitogo, C.R., Cruz-Lacierda, E.R., de la Pena, L.D., and Sunaz, N.A., 1990 : Studies on the chemical control of luminous bacteria *Vibrio harveyi* and *V. splendidus* isolated from diseased *Penaeus monodon* larvae and rearing water. *Diseases of Aquatic Organisms,* **9**, 133−139.

Bell, T.A.and Lightner, D.V., 1988 : A handbook of normal penaeid shrimp histology. World Aquaculture Society, Louisiana, 114pp.

Egusa, S., Takahashi, Y., Itami, T., and Momoyama, K., 1988 : Histopathology of vibriosis in the kuruma prawn, *Penaeus japonicus* Bate. *Fish Pathology,* **23**, 59−65.

Flegel, T. W., Fegan, D. F., Kongsom, S., Vuthikomudomkit, S., Sriurairatana, S., Boonyaratpalin, S., Chantanachookhin, C., Vickers, J. E., and Macdonald, O. D., 1992 : Occurrence, diagnosis, and treatment of shrimp diseases in Thailand, in "Diseases of cultured penaeid shrimp in Asia and the United States" (ed. by Fulks, W. and Main, K. L.), The Oceanic Institute, Hawaii, pp.57−112.

Karunasagar, I., Otta, S. K., and Karunasagar, I., 1998 : Disease problems affecting cultured penaeid shrimp in India. *Fish Pathology,* **33**, 413−419.

Lavilla-Pitogo, C.R., Baticados, M.C.L., Cruz-Lacierda, E. R., and de la Pena, L.D., 1990 : Occurrence of luminous bacterial disease of *Penaeus monodon* larvae in the Philippines. *Aquaculture,* **91**, 1−13.

Lavilla-Pitogo, C.R.and de la Pena, L.D., 1998 : Bacterial diseases in shrimp (*Penaeus monodon*) culture in the Philippines. *Fish Pathology,* **33**, 405−411.

Lester, L. J.and Pante, M. J., 1992 : Penaeid temperature and salinity responses, in "Marine Shrimp Culture : Principles and Practices" (ed. by Fast, A. W. and Lester, L. J.), Developments in Aquaculture and Fisheries Science, Vol. 23, Elsevier, Amsterdam, pp.515−534.

Lightner, D.V., 1988 : Vibrio disease of penaeid shrimp, in "Disease Diagnosis and Control in North American Marine Aquaculture" (ed. by Sindermann, C. J. and Lightner, D. V.), Developments in Aquaculture and Fisheries Science, Vol. 17, Elsevier, Amsterdam, pp.42−47.

Lightner, D. V., 1996 : Vibriosis-histological diagnostic techniques, in "A handbook of shrimp pathology and diagnostic procedures for diseases of cultured penaeid" (ed. by Lightner, D. V.), The World Aquaculture Society, Louisiana.

Wago, H., 1986 : Host defense mechanism of invertebrates from the phylogenetic viewpoint. *Protein, Nucleic acid, and Enzyme,* **31**, 886−903 (in Japanese).