Induction of Metamorphosis in the Pediveliger Larvae of the Mussel *Mytilus galloprovincialis* by Neuroactive Compounds

Cyril Glenn Satuito,*¹ Kazuyo Natoyama,*¹ Mizue Yamazaki,*²
Katsuhiko Shimizu,*³ and Nobuhiro Fusetani*⁴

Fusetani Biofouling Project, ERATO, JST

(Received September 25, 1998)

Effects of neuroactive compounds on the metamorphosis of pediveliger larvae of the mussel *Mytilus galloprovincialis* have been examined. Among the compounds tested, carbamylcholine and epinephrine both induced high percentage metamorphosis to post larvae. Norepinephrine, L-DOPA and serotonin were less active inducers of metamorphosis. These inducers were found effective at 10 μ M, except for serotonin which significantly induced metamorphosis at 100 μ M. By contrast, L-DOPA exhibited toxicity at 100 μ M. Efficacy of the inducers varied with exposure time. Epinephrine was a fast acting inducer of metamorphosis; 3 h exposure efficiently yielded maximum results. On the other hand, efficacy of carbamylcholine as inducer increased with longer exposure time.

Among the 5 vertebrate adrenergic antagonists tested, phentolamine significantly blocked the metamorphosis inducing activity of epinephrine. However, WB4104, yohimbine and propranolol all exhibited agonist activity in the absence of epinephrine.

These results suggest that use of neuroactive compounds may provide an effective and inexpensive method to control settlement and metamorphosis of mussels.

Key words: *Mytilus galloprovincialis*, metamorphosis, pediveligers, epinephrine, carbamylcholine, phentolamine

The process of settlement and metamorphosis in larvae of benthic marine invertebrates is controlled by various endogenous and exogenous factors.^{1,2)} The pediveliger larva of the mussel, upon attaining its competence, metamorphoses to the juvenile following contact and settlement by byssus thread to suitable substrata. In both field and laboratory, *Mytilus edulis* larvae have been reported to settle most readily to filamentous substrata, especially various filamentous algae.^{3,4,5)} On the other hand, Satuito *et al.*^{6,7)} claimed that larval metamorphosis could be induced by exposing pediveligers of *Mytilus galloprovincialis* to bacterial films or bacterial supernatants in the absence of filamentous substratum. Cooper⁸⁾ focused on the role of chemical cues in *M. edulis* larval metamorphosis using seawater extracts of *Platythamnion villosum* (red filamentous alga). He also demonstrated that pediveligers of the *M. edulis* settle and metamorphose in response to L-3,4-dihydroxyphenylalanine (L-DOPA), suggesting that larvae will settle and metamorphose in response to phenolic compounds, particularly those containing catechol group.⁸⁾ Nevertheless, the different factors governing settlement and metamorphosis in mussel larvae still remain unknown.

In the laboratory, exposure of larvae to neuroactive compounds has been demonstrated to effectively induce settle-

ment behavior and metamorphosis in several marine invertebrate species. Pawlik⁹⁾ suggested that these compounds interact with the nervous system rather than act directly on the epithelial receptors of larvae. Recent investigations have well documented the effect of these neuroactive compounds on the settlement and metamorphosis of the oyster *Crassostrea gigas*. For example, Coon *et al.*¹⁰⁾ reported that high percentages of larvae metamorphosed in response to both epinephrine and norepinephrine. Furthermore, these authors proposed that L-DOPA stimulates the settlement behavior in oysters through a dopamine-mediated pathway, while epinephrine may by-pass this step to interact directly with adrenergic receptors, therefore activating the morphogenic changes associated with metamorphosis.¹¹⁾ More recently, Beiras and Widdows¹²⁾ implicated the role of acetylcholine in the oyster larval settlement response, including the gross morphological changes and the reorientation of organs associated with the process of metamorphosis.

Larvae of different species respond differently to different chemical inducers. In this study, we tested various neuroactive compounds at different concentrations and exposure times on pediveliger larvae of the mussel *M. galloprovincialis*, to evaluate the ability of these compounds to induce metamorphosis. From the findings, we also dis-

*¹ Present address: Japan NUS Co. Ltd., Environmental Science Lab. 5F, Yokohama Kanazawa High Tech Center, Fukuura, Kanazawa, Yokohama 236-0004, Japan.

*² Present address: Marine Biological Association of the UK, Citadel Hill, Plymouth PL1 2PB, UK.

*³ Present address: Department of Molecular, Cellular and Developmental Biology, Marine Science Institute, University of California, Santa Barbara, California 93106, USA.

*⁴ Present address: Laboratory of Aquatic Natural Products Chemistry, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Bunkyo, Tokyo 113-8657, Japan.

cuss the role of neurotransmitter candidates in the metamorphosis of *M. galloprovincialis* pediveligers.

Materials and Methods

Larval Culture

Adult *Mytilus galloprovincialis*¹³⁾ used for spawning were collected from either Aburatsubo Bay or Nagai Port on the Miura Peninsula, Kanagawa or from Nomozaki Bay, Nagasaki, Japan. Spawning and culture methods used were as previously described.^{6,14)} Fertilized eggs, derived through artificially induced spawning, were washed with seawater and left undisturbed for 2 days. After 2 days, swimming straight hinged veliger larvae were collected, washed and cultured at an initial density of 5 larvae/ml on a diet of *Isochrysis galbana* at a concentration of 1.5×10^5 cells/ml. Water temperature was maintained at $18 \pm 1^\circ\text{C}$ under a light intensity of approximately 80 lux. Seawater used for cultures was cartridge filtered (Micro-wynd II, pore size = $0.45 \mu\text{m}$) diluted seawater (ca. 27‰ salinity). Larvae reached the pediveliger stage within 16 to 20 days from the start of culture. Only pediveligers which were 28 to 33 days old from the start of culture were employed in all assays.

Chemical Compounds

Acetylcholine chloride, carbamylcholine chloride, dopamine, epinephrine, norepinephrine, octopamine and serotonin were purchased from Sigma Chemical Co. (St. Louis, MO). Vertebrate adrenergic antagonists phentolamine, propranolol, timolol, WB4101, and yohimbine were also purchased from Sigma Chemical Co. (St. Louis, MO). L-DOPA (L-3,4-dihydroxyphenylalanine) was purchased from Wako Pure Chemicals Co. (Osaka, Japan). Ten-millimolar stock solutions were prepared by dissolving the drugs in either distilled water or diluted HCl on the day of the assays. HCl alone was tested to have no effect on larval behavior until 0.0005 N. Test solutions were prepared immediately prior to assays by diluting stock solutions to the desired concentrations in $0.22 \mu\text{m}$ (Millipore) filtered seawater (FSW). Salinity of FSW was approximately 27‰.

Larval Metamorphosis Assays

All assays were conducted at 18°C using glass petri dishes ($60 \text{ mm} \phi \times 20 \text{ mm}$). Basically, 15 to 17 larvae were released in each petri dish filled with 20 ml of the test solution for designated lengths of time. Larvae were later rinsed by transferring them using glass pipettes to containers filled with FSW. Treated larvae were finally placed in petri dishes filled with 20 ml of FSW. Larvae were observed regularly under a microscope over a 96 h experimental period to monitor behavior patterns. Metamorphosis was evaluated by verification of the post larval shell growth. Data were expressed as percentage of the total number of individuals (including dead). At least 3 replicate experiments were performed for each drug treatment.

Eight neuroactive compounds were initially tested at 1, 10, and $100 \mu\text{M}$ for their effect on metamorphosis of competent larvae, by exposing larvae to the test solutions for 24 h. Effects of exposure time on larval metamorphosis were further investigated for the 5 chemical inducers identified in the initial experiment. Larvae were exposed for

varying time periods to $100 \mu\text{M}$ of the chemical inducers, while L-DOPA was tested at $10 \mu\text{M}$. After exposure within the designated time period, larvae were rinsed as described above. Effect of treatments on larval metamorphosis was assessed using Kruskal-Wallis test, followed by pairwise comparisons between any two variables using Mann-Whitney *U* test.

Furthermore, effects of 5 vertebrate adrenergic antagonists on larval metamorphosis induced by epinephrine, identified in initial experiment as a highly active inducer, were also tested basically following the method described by Coon and Bonar.¹⁵⁾ Larvae were initially exposed to test solutions containing various concentrations of the antagonists. After a 15-min initial exposure to the antagonists, epinephrine was added into the test solution for a final concentration of $100 \mu\text{M}$. Larvae were left in the bath containing the antagonists and epinephrine for 3 h, then rinsed and transferred to petri dishes filled with FSW. Three-hour exposure time to $100 \mu\text{M}$ epinephrine was used because it was found to be most effective in consistently inducing high percentage metamorphosis. In a similar manner, larvae were exposed to $100 \mu\text{M}$ of the antagonists but without the addition of epinephrine, to check for any effect including toxicity of the drugs during the 3 h exposure time. Analysis of variance (ANOVA) was used to detect significant effect of adrenergic antagonists on the inhibition of metamorphosis induced by epinephrine. Pairwise comparisons between any two variables were assessed using Tukey-Kramer multiple comparison test. Homogeneity of variances was confirmed using Bartlett's test.

Results

Identification of Neuroactive Compounds with Metamorphosis Inducing Activity

The percentages of post larvae obtained after exposure of pediveligers to the different chemical treatments are depicted in Fig. 1. Among the 8 chemicals tested, carbamylcholine, L-DOPA, serotonin, norepinephrine, and epinephrine significantly induced metamorphosis to post larvae ($P < 0.05$), although these inducers showed different levels of activity. Inductive activity was observed at $10 \mu\text{M}$ for the inducers except for serotonin, which significantly induced metamorphosis at $100 \mu\text{M}$. Dopamine did not significantly induce larvae to metamorphosis ($P = 0.15$). Furthermore, no metamorphosis was observed in larvae exposed to acetylcholine, octopamine, and FSW.

Carbamylcholine treatment yielded near maximal results within the first 48 h upon commencement of experiment. For the other chemical inducers, maximal results were achieved only after 96 h from start of the experiment. Thereafter, observations were made until 96 h in order to obtain maximal metamorphosis.

Carbamylcholine and epinephrine were highly effective inducers of metamorphosis, while serotonin and norepinephrine were less effective. At $10 \mu\text{M}$, L-DOPA induced more than 40% metamorphosis, which was consistent with the previous report of Cooper.⁸⁾ However, this inducer showed toxicity at $100 \mu\text{M}$; more than 95% of the larvae died after exposure for 24 h.

Fig. 1. Percentages of post larvae obtained by 24-h exposure to different neuroactive compounds.

*High mortality was observed at 100 μ M of L-DOPA.

O, indicates concentrations which are significantly different from FSW ($P < 0.05$, Mann-Whitney U test).

Effect of Exposure Time on Metamorphosis

The percentage of post larvae obtained using different exposure times to the 5 chemical inducers are depicted in Fig. 2. The drugs were tested at 100 μ M except for L-DOPA, which was tested at 10 μ M.

Longer exposure time to carbamylcholine significantly yielded higher percentage of post larvae. Maximum metamorphosis was induced by exposure to the drug for 24 h, the longest exposure time tested. A similar tendency was also observed for the less effective inducers, L-DOPA, serotonin, and norepinephrine, wherein longer exposure time yielded higher percentage of post larvae. On the other hand, 30 minutes exposure to epinephrine induced more than 40% metamorphosis and maximum metamorphosis was obtained by exposure for 3 h.

Effects of Selected Vertebrate Adrenergic Antagonists on Metamorphosis

The percentages of post larvae obtained after exposure to the 5 selected vertebrate adrenergic antagonists, in both cases with and without epinephrine are depicted in Fig. 3. Among the 5 compounds tested, only phentolamine significantly blocked metamorphosis induced by epinephrine. However, 1 to 2 dead larvae were found in 2 out of 4 replicate experiments, when larvae were exposed to 100 μ M

Fig. 2. Percentages of post larvae obtained by different exposure time to the neuroactive compounds.

Results shown are those after 96 h from commencement of experiment.

Error bars indicate standard deviation (S.D.).

Fig. 3. Percentages of post larvae obtained by exposure to different concentration of vertebrate adrenergic antagonists, in cases with 100 μ M epinephrine (striped boxes) and without epinephrine (unshaded boxes).

The symbols α , α_1 , α_2 and β in parentheses represent the vertebrate adrenergic selectivity of the compounds tested.

Results shown are those after 96 h from commencement of experiment.

Error bars indicate standard deviation (S.D.).

O, indicate groups which are significantly different from the group exposed only to 100 μ M epinephrine ($P < 0.05$, Tukey-Kramer multiple comparison test).

phentolamine in the case with 100 μ M epinephrine. No mortality was observed when larvae were exposed solely to 100 μ M phentolamine. The other 4 compounds did not show any antagonist effect on metamorphosis induced by epinephrine. On the contrary, WB4101, yohimbine, and propranolol exhibited agonist activity in the absence of epinephrine; these drugs induced high percentage metamorphosis to post larvae at 100 μ M concentration. No metamorphosis was observed when larvae were exposed solely to 100 μ M timolol.

Discussion

Neuroactive compounds are known to induce settlement and/or metamorphosis in a wide range of marine invertebrate species. In molluscs, various neuroactive compounds have been reported as effective inducers. For example, L-DOPA and catecholamines have been used as inducers of metamorphosis in several bivalves (e.g. *M. edulis*,⁸ *Crassostrea* spp.,^{10,12,16} *Patinopecten yessoensis*,¹⁷ *Pecten maximus*¹⁸), GABA (γ -aminobutyric acid) in some gastropods (e.g. *Haliotis* spp.,¹⁹ *Trochus nilotica*,²⁰ *Hermissenda crassicornis*²¹), choline in opisthobranch gastr-

opods (e.g. *Phestilla sibogae*,²²⁻²⁴ *Adalaria proxima*,²⁵ *H. crassicornis*²¹), and serotonin in *C. gigas*,¹² *H. crassicornis*²¹ and *Ilyanassa obsoleta*.²⁶

In the present investigation, we found that the pediveliger of the mussel *M. galloprovincialis* can be induced to metamorphosis to post larvae by exposure to the neuroactive compounds epinephrine, norepinephrine, L-DOPA, serotonin and carbamylcholine. However, dopamine, octopamine, and acetylcholine did not induce metamorphosis in *M. galloprovincialis* pediveligers. Larval response varied with the chemical inducer. Epinephrine and carbamylcholine exhibited extremely high activity among the metamorphosis inducers identified, both often inducing 100% metamorphosis to post larvae. On the other hand, norepinephrine, L-DOPA, and serotonin were less effective inducers of metamorphosis. L-DOPA was also found toxic at a concentration of 100 μ M, while it induced metamorphosis at the lower concentration tested. Furthermore, efficacy of inducers were dependent on concentration and exposure time.

For *M. galloprovincialis*, epinephrine was found to be an effective and fast acting inducer of metamorphosis; brief exposure (3 h) of larvae to 100 μ M epinephrine solution constantly yielded high percentages of post larvae. The brief exposure time needed to induce metamorphosis suggests that metabolism of the inducer to an active form may not be required for the response.^{10,23}

The inductive activity of epinephrine was blocked by exposure of *M. galloprovincialis* larvae to phentolamine, an alpha-adrenergic antagonist, although the high concentration (100 μ M) of phentolamine caused toxic effect during prolonged exposure. However, the more selective alpha-adrenergic antagonists, WB 4101 (α_1) and yohimbine (α_2), were not only ineffective in blocking metamorphosis induced by epinephrine, but these 2 compounds also exhibited agonist activity in the absence of epinephrine and induced metamorphosis. The 2 beta-adrenergic antagonists, propranolol and timolol, also proved either ineffective or exhibited agonist activity in the absence of epinephrine. Coon and Bonar¹⁵ reported that in *C. gigas*, inductive activity of epinephrine was also blocked by phentolamine, along with other alpha-adrenergic antagonists. They also pointed out that none of the antagonists they tested demonstrated any agonist activity in the absence of epinephrine.¹⁵ One possible explanation for this difference in results could be that *M. galloprovincialis* larvae were exposed to the antagonists for longer time period as compared to the *C. gigas* larvae. Nevertheless, this warrants further investigation.

In the oyster *C. gigas*, epinephrine is a fast and efficient inducer of metamorphosis,¹⁰ which can be blocked by adrenergic antagonists.¹⁵ In addition, norepinephrine is found present in larval tissues.²⁷ These findings were basis for the hypothesis by Bonar *et al.*¹¹ that epinephrine may act directly on larval adrenergic receptors which mediate the endogenous metamorphic events in oyster larvae. Our findings suggest that epinephrine may act directly on the larval adrenergic receptors during metamorphosis in *M. galloprovincialis*. This is also supported by the existence of catecholamines in pediveligers of *M. galloprovincialis* as proven by glyoxilic acid staining (own unpublished observation).

Another metamorphosis inducer identified in this investigation was carbamylcholine, which induced high percentage metamorphosis at concentrations between 10 to 100 μM . This compound was found ineffective on larvae of the polychaete *Phragmatopoma lapidosa californica* even at a concentration of 10 mM.⁹⁾ The effective concentrations of carbamylcholine found in the present investigation are also much lower than previous reports on the related compound, choline, which induced metamorphosis in opisthobranch gastropods at concentrations greater than millimolar.²¹⁻²³⁾ Carbamylcholine is an agonist of the neurotransmitter acetylcholine, and is not hydrolyzed by the enzyme acetylcholinesterase. Parallel tests conducted with carbamylcholine indicated that acetylcholine does not induce metamorphosis in pediveligers of *M. galloprovincialis*. Raineri²⁸⁾ reported the presence of acetylcholinesterase activity in larvae of *M. galloprovincialis*. Our findings therefore suggest that acetylcholinesterase-activity may have blocked the effect of externally applied acetylcholine through hydrolysis. Coon *et al.*¹⁰⁾ reported low or inconsistent inductive activity of acetylcholine for *C. gigas*. By contrast, Beiras and Widdows¹²⁾ induced settlement behavior, cementation, and eventual metamorphosis by exposing oyster larvae to acetylcholine, and yielded post larvae which were all settled to substrata. The role of acetylcholine during metamorphosis of *M. galloprovincialis* still requires further investigation.

Previous studies have already demonstrated that pediveligers of mussels (*M. edulis*) and oysters (*C. gigas*) undergo metamorphosis in response to L-DOPA^{8,10,12)} and serotonin.¹²⁾ In the present investigation, L-DOPA and serotonin also induced metamorphosis in *M. galloprovincialis* larvae, although L-DOPA was toxic at the higher concentration (100 μM). Toxicity of 100 μM L-DOPA in oyster larvae was also reported by Beiras and Widdows.¹²⁾

Bonar *et al.*¹¹⁾ proposed that for oyster larvae, control of settlement behavior appears to be through a dopaminergic receptor-mediated pathway. They reported that externally applied L-DOPA triggers a distinctive pattern of settlement behavior in oyster larvae, and proposed that L-DOPA is converted into dopamine within the larval body which then acts through the dopaminergic receptors.¹¹⁾

In *M. galloprovincialis* larvae which have been exposed to L-DOPA, we could not observe any distinctive behavioral pattern that can be associated with settlement (e.g. crawling, settlement by byssus thread). Instead, we observed that upon prolonged exposure (24 h) to L-DOPA, larvae became entangled in mucus-like materials secreted on the bottom surface of petri dishes by the larvae themselves. We also observed that these larvae eventually metamorphosed to the post larval stage. Beiras and Widdows¹²⁾ also noted mucus secretions of their larvae when exposed to dopamine. In *M. galloprovincialis*, the role of L-DOPA as a metamorphosis inducer remains to be clarified.

Neuroactive compounds, specifically epinephrine and carbamylcholine, can provide a simple, inexpensive and reliable method of inducing high percentage metamorphosis in pediveliger larvae of *M. galloprovincialis*. Similarly, neuroactive compounds may find application in the development of techniques to control settlement and metamorphosis of larval *M. galloprovincialis*.

Acknowledgments We wish to express our gratitude to Dr. H. B. Koh, to Mr. H. Funatsuki and to the staff of Misaki Marine Biological Station, University of Tokyo, for their assistance in collection of mussels. We also thank Dr. K. Okano (RIKEN) and Dr. S. Kawai (BRAIN) for discussions.

References

- 1) D. J. Crisp: Factors influencing the settlement of marine invertebrate larvae, in "Chemoreception in Marine Organisms" (ed. by P. T. Grant and A. M. Mackie), Academic Press, New York, 1974, pp. 177-265.
- 2) J. R. Pawlik: Chemical ecology of the settlement of benthic marine invertebrates. *Oceanogr. Mar. Biol. Annu. Rev.*, **30**, 273-335 (1992).
- 3) B. L. Bayne: Growth and delay of metamorphosis of the larvae of *Mytilus edulis* L. *Ophelia*, **2**, 1-47 (1965).
- 4) B. L. Bayne: Primary and secondary settlement in *Mytilus edulis* L. (Mollusca). *J. Anim. Ecol.*, **33**, 513-523 (1964).
- 5) L. S. Eyster and J. A. Pechenik: Attachment of *Mytilus edulis* L. larvae on algal and byssal filaments is enhanced by water agitation. *J. Exp. Mar. Biol. Ecol.*, **114**, 99-110 (1987).
- 6) C. G. Satuito, K. Natoyama, M. Yamazaki, and N. Fusetani: Induction of attachment and metamorphosis of laboratory cultured mussel *Mytilus edulis galloprovincialis* by microbial film. *Fisheries Sci.*, **61**, 223-227 (1995).
- 7) C. G. Satuito, K. Shimizu, and N. Fusetani: Studies on the factors influencing larval settlement in *Balanus amphitrite* and *Mytilus galloprovincialis*. *Hydrobiologia*, **358**, 275-280 (1997).
- 8) K. Cooper: A model to explain the induction of settlement and metamorphosis of planktonic eyed-pediveligers of the blue mussel *Mytilus edulis* L. by chemical and tactile cues. *J. Shellfish Res.*, **2**, 117 (1982).
- 9) J. R. Pawlik: Natural and artificial induction of metamorphosis of *Phragmatopoma lapidosa californica* (Polychaeta: Sabellariidae), with a critical look at the effect of bioactive compounds on marine invertebrate larvae. *Bull. Mar. Sci.*, **46**, 512-536 (1990).
- 10) S. L. Coon, D. B. Bonar, and R. M. Weiner: Induction of settlement and metamorphosis of the Pacific oyster, *Crassostrea gigas* (Thunberg), by L-DOPA and catecholamines. *J. Exp. Mar. Biol. Ecol.*, **94**, 211-221 (1985).
- 11) D. B. Bonar, S. L. Coon, M. Walch, R. M. Weiner, and W. Fitt: Control of oyster settlement and metamorphosis by endogenous and exogenous chemical cues. *Bull. Mar. Sci.*, **46**, 484-498 (1990).
- 12) R. Beiras and J. Widdows: Induction of metamorphosis in larvae of the oyster *Crassostrea gigas* using neuroactive compounds. *Mar. Biol.*, **123**, 327-334 (1995).
- 13) K. Inoue, S. Odo, T. Noda, S. Nakao, S. Takeyama, E. Yamaha, F. Yamazaki, and S. Harayama: A possible hybrid zone in the *Mytilus edulis* complex in Japan revealed by PCR markers. *Mar. Biol.*, **128**, 91-95 (1997).
- 14) C. G. Satuito, K. Natoyama, M. Yamazaki, and N. Fusetani: Larval development of the mussel *Mytilus edulis galloprovincialis* cultured under laboratory conditions. *Fisheries Sci.*, **60**, 65-58 (1994).
- 15) S. L. Coon and D. B. Bonar: Pharmacological evidence that alpha1-adrenoceptors mediate metamorphosis of the Pacific oyster, *Crassostrea gigas*. *Neuroscience*, **23**, 1169-1174 (1987).
- 16) S. H. Tan and T. M. Wong: Induction of settlement and metamorphosis in the tropical oyster, *Crassostrea belcheri* (Sowerby), by neuroactive compounds. *J. Shellfish Res.*, **14**, 435-438 (1995).
- 17) B. C. Kingzett, N. Bourne, and K. Leask: Induction of metamorphosis of the Japanese scallop *Patinopecten yessoensis* Jay. *J. Shellfish Res.*, **9**, 119-124 (1990).
- 18) L. Chevolot, J. C. Cochard, and J. C. Yvin: Chemical induction of larval metamorphosis of *Pecten maximus* with a note on the nature of naturally occurring triggering substances. *Mar. Ecol. Prog. Ser.*, **74**, 83-89 (1991).
- 19) D. E. Morse: Biochemical and genetic engineering for improved production of abalones and other valuable molluscs. *Aquaculture*, **39**, 263-282 (1984).
- 20) E. Tamashiro, Y. Fujita, and K. Sakata: Induction of larval

- metamorphosis of *Trochus nilotica* by γ -aminobutyric acid and a coralline red algal extract. *Nippon Suisan Gakkaishi*, **59**, 1261 (1993).
- 21) C. Avila, C. Tamse, and A. M. Kuzirian: Induction of metamorphosis in *Hermisenda crassicornis* larvae (Mollusca: Nudibranchia) by GABA, choline and serotonin. *Invertebrate Reprod. Develop.*, **29**, 127-141 (1996).
- 22) M. G. Hadfield: Settlement requirement of molluscan larvae: new data on chemical and genetic roles. *Aquaculture*, **39**, 283-298 (1984).
- 23) M. G. Hadfield: Metamorphosis in molluscan larvae: an analysis of stimulus and response, in "Settlement and Metamorphosis of Marine Invertebrate Larvae" (ed. by F. S. Chia and M. E. Rice), Elsevier, New York, 1978, pp. 165-175.
- 24) K. Y. Hirata and M. G. Hadfield: The role of choline in metamorphic induction of *Phestilla* (Gastropoda, Nudibranchia). *Comp. Biochem. Physiol.*, **84C**, 15-21 (1986).
- 25) C. Todd, M. G. Bentley, and J. N. Havenhand: Larval metamorphosis of the opisthobranch mollusc *Adalaria proxima* (Gastropoda: Nudibranchia): the effects choline and elevated potassium ion concentration. *J. Mar. Biol. Ass. U.K.*, **71**, 53-72 (1991).
- 26) J. M. Couper and E. M. Leise: Serotonin injections induce metamorphosis in larvae of the gastropod mollusc *Ilyanassa obsoleta*. *Biol. Bull.*, **191**, 178-186 (1996).
- 27) S. L. Coon and D. B. Bonar: Norepinephrine and dopamine content of larvae and spat of the pacific oyster, *Crassostrea gigas*. *Biol. Bull.*, **171**, 632-639 (1986).
- 28) M. Raineri: Is a mollusc an evolved bent metatrocophore? A histochemical investigation of neurogenesis in *Mytilus* (Mollusca: Bivalvia). *J. Mar. Biol. Ass. U. K.*, **75**, 571-592 (1995).