

**Induction of Metamorphosis of Pediveliger Larvae of the Mussel  
*Mytilus galloprovincialis* Lamarck, 1819 using Neuroactive Compounds,  
KCl, NH<sub>4</sub>Cl and Organic Solvents**

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## Abstract

Pediveliger larvae of *Mytilus galloprovincialis* were subjected to a series of bioassays to investigate the induction of metamorphosis using neuroactive compounds,  $K^+$ ,  $NH_4^+$  and organic solvents. Growth and survival of post-larvae obtained using ethanol and methanol were also observed. Epinephrine, phenylephrine, clonidine and metanephrine induced larval metamorphosis at  $10^{-6}$  -  $10^{-4}$  M in both 24-h and continuous exposure assays. In 24-h exposure assays,  $\alpha$ -methyldopa at  $5 \times 10^{-5}$  M and methoxyphenamine at  $5 \times 10^{-5}$  -  $10^{-4}$  M induced 55 - 94% metamorphosis. Similarly, excess  $K^+$  at  $3 \times 10^{-2}$  M induced 39% metamorphosis and  $NH_4^+$  at  $1 - 5 \times 10^{-2}$  M induced 63 - 78% metamorphosis. EC50s of seven organic solvents ranged from 0.04 to 0.82 M. Post-larvae that metamorphosed using ethanol and methanol survived as juveniles and grew at the same rate as those from microbial biofilm. Thus, above compounds can be useful inducers of metamorphosis for antifouling studies using larvae and juveniles of *M. galloprovincialis*.

**Keywords:** *Mytilus galloprovincialis*, larval metamorphosis, artificial inducers, adrenergic agonists, organic solvents, antifouling

## INTRODUCTION

Mussels are typical members of fouling communities worldwide. The mussel, *Mytilus galloprovincialis* Lamarck, 1819, is believed to have been introduced to Japan in the late 1920's (Otani, 2002) but is now the dominant species in inter-tidal zones of most of the country (Sakaguchi, 1987; Sakaguchi & Kajihara, 1988). It is estimated that *M. galloprovincialis* accounts for approximately 80% (in biomass) of all fouling organisms on man-made structures submerged in Tokyo Bay, Japan (Kajihara, 1985). Uncontrolled growth of fouling organisms including the pervasive *M. galloprovincialis* on cooling water systems of electric power plants hinders normal operation and has costly consequences (Katsuyama, 1995; Sakaguchi, 2003). Presently, antifouling paints and chlorination are widely used in Japanese electric power stations to control biofouling (Sakaguchi, 2003). Efforts are being made to develop environmentally friendly antifouling technologies.

*M. galloprovincialis*, as with many other marine invertebrates, possess a planktonic larval phase preceding a benthic adult phase. Larvae of most marine invertebrates must recognize exogenous cues prior to their settlement and metamorphosis (see review by Crisp, 1974; Morse, 1990; Pawlik, 1992; Qian, 1999; Hadfield & Paul, 2001). In *M.*

*galloprovincialis*, larval settlement and metamorphosis have been shown to be dependent on the presence of bacteria (Satuito et al., 1995; Bao et al., 2007a) and macroalgae (Yang et al., 2007). Although it has been suggested that chemical cue(s) from these natural inducers are involved in the induction of larval settlement and metamorphosis in this species (Bao et al., 2007b; Yang et al., 2007), these cues have yet to be identified. In order to successfully develop effective antifouling measures against mussel colonization of pipes, nets and ship hulls, it is important to understand the mechanism of their larval settlement and metamorphosis (see Dobretsov & Qian, 2003).

Various compounds such as vertebrate neurotransmitter compounds (Pawlik, 1990; Beiras & Widdows, 1995; Yamamoto et al., 1996; Boettcher & Targett, 1998; Satuito et al., 1999; Dobretsov & Qian, 2003; Yu et al., 2007) and ions of  $K^+$ ,  $Cs^+$ ,  $Li^+$  and  $NH_4^+$  (Baloun & Morse, 1984; Yool et al., 1986; Degnan et al., 1997; Carpizo-Ituarte & Hadfield, 1998; Zhao et al., 2003; Li et al., 2006; Seipp et al., 2007) have been used to induce settlement and metamorphosis of larvae of hydroids, bryozoans, polychaetes, mollusks, barnacles, sea urchins and ascidians. Even sulfide and organic solvents have been reported to induce larval settlement and metamorphosis in the polychaete *Capitella* sp. I (Dubilier, 1988) and the nudibranch *Phestilla sibogae* (Pennington & Hadfield, 1989). Reported effects of neurotransmitters on larval settlement and metamorphosis

vary with species. For example, epinephrine has been shown to induce larval metamorphosis in the bivalves *Crassostrea gigas* (Coon et al., 1985; Beiras & Widdows, 1995), *Pecten maximus* (Chevolot et al., 1991; Nicolas et al., 1996), *Argopecten purpuratus* (Martinez et al., 1999) and *M. galloprovincialis* (Satuito et al., 1999; García-Lavandeira et al., 2005) but had no effect on *Balanus amphitrite* (Yamamoto et al., 1996). Excess  $K^+$  ions in seawater has also been found to induce larval metamorphosis in *A. purpuratus* but is ineffective in *M. edulis* (Eyster & Pechenik, 1987; Dobretsov & Qian, 2003).

Although the sites and mechanisms of action of these compounds are unknown, it has been suggested that these chemicals mimic substratum derived natural inducers (Yvin, 1985; Pawlik, 1986), or act directly on signalling pathways (Baloun & Morse, 1984; Coon et al., 1985; Coon & Bonar, 1987; Bonar et al., 1990; Morse, 1990). Nonetheless, researchers are investigating the effects of these chemicals on different larvae with the purpose of obtaining information that will contribute to the elucidation of the mechanism of larval settlement and metamorphosis (Coon & Bonar, 1987; Clare et al., 1995; Yamamoto et al., 1996; Holm et al., 1998; Hadfield et al., 2000). Moreover, researchers also attempt to identify suitable chemical inducer compounds that can find application in laboratory bioassay for biofouling and antifouling research and in

aquaculture (Zhao et al., 2003).

In the present study, the authors investigated the effects of neuroactive compounds, potassium chloride (KCl), ammonium chloride (NH<sub>4</sub>Cl) and organic solvents on the larval metamorphosis of *M. galloprovincialis*. The purpose was to obtain information on the response of *M. galloprovincialis* to these chemicals, which are known inducers of metamorphosis in larvae of other species, consequently identifying potential metamorphosis inducers for laboratory bioassays using *M. galloprovincialis* larvae. Growth and survival of juveniles that metamorphosed using organic solvents were also investigated.

## **MATERIALS AND METHODS**

### **Spawning and larval culture**

Adult *M. galloprovincialis* used for spawning were either collected from populations growing on the wharf adjacent to the Nagasaki Prefecture Fisheries Experimental

Station, Taira-machi, Nagasaki (129°51'E; 32°43'N), or purchased from a culture farm in Matoya, Isobe-machi, Mie (136°52'E; 34°22'N), Japan. Spawning in the laboratory was induced following basically the method described by Satuito et al. (2005). Mussels were cleaned by brushing off materials attached on the shell surfaces and rinsing in seawater, packed in ice overnight and transferred to a 30 l polycarbonate tank with filtered sea water (Whatman glass-fiber filter, GF/C: 1.2  $\mu\text{m}$ , FSW) at ca. 24°C; final water temperature was ca. 21°C. Mussels that started spawning were transferred to 2 l glass beakers and were let to spawn individually. Eggs were collected using a glass pipette in a beaker containing FSW, fertilized by gently mixing a sperm suspension in FSW and then left undisturbed for 20 min. Fertilized eggs were filtered on a nylon plankton net (mesh size: 20  $\mu\text{m}$ ) to remove excess sperm, washed thoroughly with FSW and left undisturbed for two days inside an incubator maintained at 17°C. After two days, swimming straight-hinge veliger larvae were collected, washed gently with FSW and cultured following the methods described by Satuito et al. (2005). Briefly, straight-hinge larvae were cultured in 2 l glass beakers at an initial stocking density of 5 larvae  $\text{ml}^{-1}$ . Larvae were fed a diet of *Chaetoceros gracilis* at  $5 \times 10^4$  cells  $\text{ml}^{-1}\text{day}^{-1}$ . Culture water was changed every other day and the temperature maintained at  $17 \pm 1^\circ\text{C}$ . Larvae were cultured to the pediveliger stage of growth and were used in metamorphosis bioassays

when shell height (SH) and shell length (SL) reached  $>288 \mu\text{m}$  and  $>309 \mu\text{m}$ , respectively (Satuito et al. 2005).

Straight-hinge veliger larvae were also stored inside a refrigerator for a maximum period of three months and were cultured inside an incubator to the pediveliger stage when needed in assays. This ensured the supply of pediveligers almost all year round. Refrigeration had no adverse effects on the survival, growth, settlement and metamorphosis of larvae (Satuito et al. 2005). Conditions for storing the larvae in the refrigerator were the same as that reported by Satuito et al. (2005). Conditions for culturing the refrigerated larvae were the same as described above.

### Chemical compounds

Chemical compounds used in the investigation, together with the respective manufacturers and places of purchase, are shown in Table 1. Chemicals selected for the investigation are known to be effective inducers of metamorphosis in larvae of various marine invertebrate species. Each stock solution of the seven adrenergic agonist, metanephrine, VMA and adrenochrome was prepared by dissolving the chemical in 0.22

$\mu\text{m}$  Millipore filtered seawater (0.22  $\mu\text{m}$  FSW) or initially in 0.3 to 0.5 ml of diluted HCl and then diluting it in 100 ml of 0.22  $\mu\text{m}$  FSW (pH 7.6 to 7.8). Stock solutions of KCl,  $\text{NH}_4\text{Cl}$  and the eight organic solvents were prepared by diluting these chemicals in 0.22  $\mu\text{m}$  FSW. Concentrations of stock solutions of the chemicals investigated are shown in Table 1. Test solutions assayed were prepared by diluting stock solutions in 0.22  $\mu\text{m}$  FSW to desired concentrations (Table 1). All stock solutions and test solutions were prepared on the same day of the assay.

#### Larval metamorphosis bioassays

Pediveliger larvae that were  $> 288\mu\text{m}$  in SH and  $> 309\mu\text{m}$  in SL were employed in metamorphosis bioassays. Twenty pediveliger larvae were released in each glass Petri dish ( $\text{\O} 60 \text{ mm} \times 15 \text{ mm}$  height) containing 20 ml of the test solution. Larvae were subjected to the test solution either in a 24-h exposure bath (Coon & Bonar, 1987; Satuito et al., 1999) or in continuous exposure throughout the experimental period. Larvae that were given the 24-h exposure bath were rinsed three times with 0.22  $\mu\text{m}$  FSW and then finally transferred to Petri dishes containing each of 20 ml of 0.22  $\mu\text{m}$

FSW. In both 24-h and continuous exposure assays, larvae were checked after 48 h and 72 h from the commencement of assays and evaluated for metamorphosis by verification of post-larval shell growth. Dead larvae in each Petri dish were also recorded. Data were expressed as the percentage of post-larvae from total number of individuals per Petri dish. For each chemical compound, six to 21 replicate assays from two to six different culture batches were performed. However, continuous exposure assays with  $\alpha$ -methyldopa, isoproterenol and dobutamine were conducted in only three replicates from a single culture batch of larvae. A Petri dish containing 20 larvae and 20 ml of 0.22  $\mu$ m FSW was always set in each assay as the control. All assays were conducted at  $17 \pm 1^\circ\text{C}$  in a dark environment.

Culture of post-larvae that metamorphosed using EtOH, MeOH and biofilms as metamorphosis inducers

Post-larvae that metamorphosed using EtOH, MeOH and biofilms as inducers were employed in the culture experiment. Post-larvae obtained from biofilms were used as the control group. Forty post-larvae were collected from each of the two solvents and

the biofilm, transferred to Petri dishes ( $\varnothing$  90 mm  $\times$  15 mm height) containing 30 ml of FSW, and cultured for 28 days in a dark environment at a temperature of  $17 \pm 1^\circ\text{C}$ . During the culture period, post-larvae were fed  $10 \times 10^4$  cells  $\text{ml}^{-1} \text{day}^{-1}$  of *C. gracilis* for the first nine days and were fed  $20 \times 10^4$  cells  $\text{ml}^{-1} \text{day}^{-1}$  of the algae from the 10<sup>th</sup> day. Culture water was renewed every other day during the culture period.

Survival and growth rates of post-larvae that metamorphosed from each of the three groups were recorded every week. For each group, post-larval growth was calculated from the increase in average shell length of 20 larvae.

#### Data analysis

Metamorphosis inducing activities of the chemical compounds were expressed as percentages of post-larvae. Inducing activities of adrenergic agonists, metanephrine, VMA, adrenochrome, KCl,  $\text{NH}_4\text{Cl}$  and organic solvents were assessed using the Kruskal-Wallis Test. EC50 and LC50 of organic solvents were calculated by probit analysis (Finney, 1971). Growth rates of post-larvae were assessed using with one-way Analysis of Variance (ANOVA). All statistical computations were performed using the

JMP™ software. Differences were considered significant at  $p < 0.05$ .

## RESULTS

Throughout all experiments, no post-larvae of *M. galloprovincialis* metamorphosed in the controls.

Effects of adrenergic agonists, metanephrine, VMA and adrenochrome on larval metamorphosis

Percentages of post-larvae on the seven adrenergic agonists when larvae were subjected to 24-h and continuous exposure assays are as shown in Figure 1a and 1b, respectively.

In the 24-h exposure assay, only epinephrine (Kruskal-wallis test:  $p < 0.001$ ), phenylephrine ( $p < 0.05$ ), and clonidine ( $p < 0.001$ ) exhibited inducing activities after 48 h (Figure 1a). After 72 h,  $\alpha$ -methyldopa ( $p < 0.001$ ) and methoxyphenamine ( $p < 0.001$ ) also induced post-larval metamorphosis (Figure 1a). Percentages of post-larvae

in these compounds increased with increasing concentration but  $\alpha$ -methyldopa ( $p < 0.001$ ) exhibited maximum metamorphosis at  $5 \times 10^{-5}$  M concentration. No inducing activity was observed in isoproterenol ( $p = 0.41$ ) and dobutamine ( $p = 0.11$ ) at all concentrations tested during the 72 h assay period (Figure 1a). In the continuous exposure assay, larval metamorphosis was observed in epinephrine ( $p < 0.01$ ), phenylephrine ( $p < 0.001$ ) and clonidine ( $p < 0.001$ ) after 48 h (Figure 1b). After 72 h, percentages of post-larvae in these three compounds further increased. For epinephrine, the percentage of post-larvae was highest at  $5 \times 10^{-5}$  M but decreased thereafter. Isoproterenol ( $p < 0.05$ ) induced metamorphosis only at  $10^{-5}$  M after 72 h while the other compounds showed no activity at all concentrations tested. Mortality of larvae in these chemicals ranged from 0 to  $2 \pm 3\%$ , except in the continuous exposure assay with clonidine where it is was  $8 \pm 9\%$  at  $10^{-6}$  M concentration.

Percentages of post-larvae in metanephrine, VMA and adrenochrome after 72 h when larvae were subjected to these compounds in 24-h and continuous exposure assays are shown in Figure 2. Metanephrine induced larval metamorphosis in both 24-h and continuous exposure assays (24-h exposure:  $p < 0.05$ , continuous exposure:  $p < 0.001$ , Figure 2a). In metanephrine ( $p < 0.05$ ), the percentage of post-larvae increased with increasing concentration during continuous exposure but was only  $17 \pm 10\%$  at  $10^{-4}$  M.

VMA, another epinephrine metabolite, did not induce larval metamorphosis at all concentrations tested in both 24-h ( $p = 0.28$ ) and continuous ( $p = 1$ ) exposure assays (Figure 2b). Adrenochrome, an oxidative product of epinephrine, induced  $12 \pm 8\%$  post-larvae only in the 24-h exposure assay ( $p < 0.05$ ) but was ineffective at all concentrations in the continuous exposure assay ( $p = 1$ , Figure 2c). Larval mortality of  $< 1\%$  was observed in metanephrine in both 24-h and continuous exposure assays. No mortality was observed in VMA and adrenochrome.

#### Effects of KCl and NH<sub>4</sub>Cl on larval metamorphosis

No larvae metamorphosed in KCl after 48 h in both 24-h and continuous exposure assays. Percentages of post-larval metamorphosis in different concentrations of KCl after 72 h are as shown in Figure 3a. Thirty-millimolar of KCl induced  $39 \pm 14\%$  metamorphosis to post-larvae but higher concentration induced  $16 \pm 6\%$  larval metamorphosis in the 24-h exposure assay (Kruskal-wallis:  $p < 0.001$ , Figure 3a). By contrast, continuous exposure to KCl did not induce larval metamorphosis (Figure 3a), even though some larvae at  $3 \times 10^{-2}$  M and higher concentrations were observed lying

with extended foot during the assay period. No mortality of larvae was observed in both 24-h and continuous exposure assays of KCl.

No metamorphosis was observed in  $\text{NH}_4\text{Cl}$  in 24-h and continuous exposure assays after 48 h. Percentages of larval metamorphosis in different concentrations of  $\text{NH}_4\text{Cl}$  after 72 h are as shown in Figure 3b. After 72 h, larval metamorphosis was observed only in the 24-h exposure assay of  $\text{NH}_4\text{Cl}$ ; with > 60% metamorphosing to post-larvae at  $10^{-2}$  M and higher concentrations (Kruskal-wallis:  $p < 0.001$ , Figure 3b). During the continuous exposure assay, some larvae at  $10^{-2}$  M and higher concentrations were observed lying with extended foot. In the case of  $\text{NH}_4\text{Cl}$ , no larval mortality was observed in the 24-h exposure assay and mortalities were < 5% at  $10^{-2}$  M and higher concentrations in the continuous exposure assay.

#### Larval metamorphosis inducing activities of organic solvents

No larvae metamorphosed after 48 h in the 24-h and continuous exposure assays of the eight organic solvents. Percentages of post-larvae in the eight different organic solvents after 72 h in 24-h and continuous exposure assays are shown in Figure 4. All eight

organic solvents induced larval metamorphosis in the 24-h exposure assay. EtOH, MeOH, EG, ACN, DMSO and Ace induced maximum metamorphosis at concentrations between 0.5 and 1.0 M, while nPrOH induced  $92 \pm 4\%$  metamorphosis to post-larvae at 0.1 M. Percentage of post-larvae in hx in the 24-h exposure assay was constantly  $< 30\%$  until 3.0 M concentration. By contrast, no metamorphosis was observed in all eight organic solvents at all concentrations tested in the continuous exposure assay even after 72 h (Figure 4). In both 24-h and continuous exposure assays of these solvents, some larvae were observed lying with extended foot while others were apparently with impaired velum after 24 h from the start of assays. Mortalities on these eight solvents in both assays were concentration dependent.

Based on results of the 24-h exposure assay, concentrations to induce 50% metamorphosis (EC50) and 50% mortalities (LC50) of the eight organic solvents were calculated as shown in Table 2. EC50s of EtOH, MeOH, EG, ACN, DMSO and Ace ranged from 0.37 to 0.82 M and their LC50s ranged from 1.02 to 2.08 M (Table 2). On the other hand, nPrOH had the lowest EC50 among the eight solvents tested and was also the most toxic, while hx, which showed the lowest inducing activity, was the least toxic solvent used.

## Growth and survival of post-larvae that metamorphosed using EtOH and MeOH as inducers

Increases in SL during the four-week culture of post-larvae that metamorphosed using EtOH, MeOH and biofilm are as shown in Figure 5. After four weeks of culture, average SL reached 1477  $\mu\text{m}$ , 1695  $\mu\text{m}$  and 1662  $\mu\text{m}$  for post-larvae obtained from biofilm, EtOH and MeOH groups, respectively. Daily growth (in SL) and survival rates after one month culture of post-larvae that metamorphosed using the three inducers are summarized in Table 3. During the first two weeks of culture, average growth rates were 41  $\mu\text{m d}^{-1}$ , 46  $\mu\text{m d}^{-1}$  and 44  $\mu\text{m d}^{-1}$  for post-larvae from biofilms, EtOH and MeOH groups, respectively, and no significant difference was observed in growth rates between the three groups (ANOVA:  $p > 0.05$ , Table 3). Similarly, growth rates of the three groups were the same during the latter two weeks of the culture (ANOVA:  $p > 0.05$ ) and ranged from 63  $\mu\text{m d}^{-1}$  to 85  $\mu\text{m d}^{-1}$ . A 100% survival of post-larvae was observed in the three groups during the four-week culture period (Table 3).

## DISCUSSION

Although the study in the area of marine invertebrate settlement cue has a long history, only a few natural chemical inducers have been identified to date (Yvin et al., 1985; Pawlik, 1986). Researchers therefore resort to the use of known compounds that are commercially available (e.g. neuroactive compounds, ions, etc.) in their attempt to explain the mechanism of larval settlement (Coon & Bonar, 1987; Pawlik, 1990; Clare et al., 1995; Boettcher & Targett, 1998; Biggers & Laufer, 1999; Hadfield et al., 2000). In addition, researchers have also tested these compounds for effectiveness in controlling settlement and metamorphosis for biofouling and aquaculture purposes (Cooper, 1983; Martinez et al., 1999; Doroudi & Southgate, 2002; Dobretsov & Qian, 2003; Gapasin & Polohan, 2004; García-Lavandeira et al., 2005; Yu et al., 2007). In the present investigation, the authors have demonstrated that chemical compounds such as epinephrine, phenylephrine, clonidine, KCl, NH<sub>4</sub>Cl and organic solvents induce mussel larval metamorphosis.

Previous studies have already shown that epinephrine induce *M. galloprovincialis* larval metamorphosis (Satuito et al., 1999; García-Lavandeira et al.,

2005). These researchers (Satuito et al., 1999) further discussed that epinephrine may act directly on the nervous system of *M. galloprovincialis* larvae to induce metamorphosis. Results in the present investigation showed that other vertebrate adrenergic agonists, particularly phenylephrine and clonidine, also induce larval metamorphosis. Previous reports (Yamamoto et al., 1998; Satuito et al., 1999; 2005) have also demonstrated that phentolamine, an adrenergic antagonist, inhibits *M. galloprovincialis* larval metamorphosis. These observations suggest that receptors mediating *M. galloprovincialis* larval metamorphosis may have similarities to the vertebrate-type alpha adrenoceptors. Consequently, *M. galloprovincialis* larval metamorphosis can be effectively controlled by using adrenergic agonist and antagonist compounds. Bacq (1949) and Pawlik (1990) reported that catecholamines are subject to oxidation and polymerization in solution. In the present investigation, metanephrine, VMA and adrenochrome all exhibited low (< 20%) or even no inductive activity, implying that epinephrine, and not the metabolized or oxidized product, may be the active form required for larval response. Moreover, epinephrine has been demonstrated to give maximal results even at short exposure time for *M. galloprovincialis* (Satuito et al., 1999) and *C. gigas* (Coon & Bonar, 1987) larvae.

Elevated potassium at concentrations ranging from  $10^{-3}$  to  $7.5 \times 10^{-2}$  M has

been reported to induce larval metamorphosis in a wide variety of species, e.g. polychaetes *Capitella* sp. I (Biggers & Laufer, 1999), *Phragmatopoma californica* (Yool et al., 1986) and *Hydroides elegans* (Carpizo-Ituarte & Hadfield, 1998), mollusks *P. sibogae* (Yool et al., 1986), *A. purpuratus* (Martinez et al., 1999), *Haliotis diversicolor supertexta* (Li et al., 2006), bryozoan *B. neritina* (Yu et al., 2007), sea urchin *Lytechinus variegatus* (Cameron et al., 1989) and ascidian *Herdmania momus* (Degnan et al., 1997). The result of the present study adds *M. galloprovincialis* to the list of species that metamorphose in response to exposure to elevated potassium. However, the inductive activity of KCl was lower (ca. 40%) than the other compounds tested in the present study. Moreover, no metamorphosis was observed in the present study when larvae were exposed continuously to elevated potassium and this observation is consistent with those reported for *M. edulis* larvae (Eyster & Pechenik, 1987; Dobretsov & Qian, 2003). Potassium is known to act by depolarizing excitable cells involved in the perception of stimulus or by directly activating the larval nervous system (Baloun & Morse, 1984; Yool et al., 1986; Pawlik, 1990; Martinez et al., 1999; Zhao et al., 2003). In the present study, merely exposing larvae to excess KCl may have not been sufficient to induce metamorphosis but transferring treated larvae to FSW after 24 h could have physiologically “shocked” larvae and thus triggered metamorphosis. Nevertheless, this

warrants further investigation.

$\text{NH}_4^+$ , an effective inducer of settlement in the oysters *C. gigas* (Bonar et al., 1990; Coon et al., 1990; Fitt & Coon, 1992) and *C. virginica* (Fitt & Coon, 1992) and metamorphosis in the ascidian *Ciona intestinalis* (Berking & Herrmann, 1990), induced high percentage of larval metamorphosis in the present study. In *M. galloprovincialis*, the induction of larval metamorphosis by this ion was similar to excess  $\text{K}^+$ , in that post-larvae were only observed after larvae were transferred to FSW from the  $\text{NH}_4^+$  solution. However, some larvae in the lower concentration group ( $\leq 10^{-2}$  M) were also observed lying with extended foot while in the test solution; a behavior also exhibited by *C. gigas* in  $\text{NH}_4^+$  solution and evaluated as settlement related by Coon et al. (1990). For *M. galloprovincialis*,  $\text{NH}_4^+$  can therefore be a promising inducer of larval metamorphosis.

The present investigation also demonstrated that organic solvents induce larval metamorphosis in *M. galloprovincialis*. Pennington & Hadfield (1989) were the first to induce larval metamorphosis of the nudibranch *P. sibogae* using different organic solvents including those tested in the present study. Solvents found effective in the present study also induced *P. sibogae* larval metamorphosis (Pennington & Hadfield, 1989), except for EG and DMSO. In general, effective concentrations were also similar

for the two species (Pennington & Hadfield, 1989; this study). In natural situations, organic solvents do not exist in the seawater, especially in concentrations used in the present investigation and therefore have no ecological relevance. Although the mechanism of action of organic solvents on larvae during metamorphosis is unknown, Pennington & Hadfield (1989) suggested that solvents probably penetrate larval tissues and interfere with a wide range of nervous activities, somehow activating the metamorphic pathway. Another explanation could be that sub-toxic levels of these solvents could have triggered larval metamorphosis by physiologically “shocking” the larvae since dead larvae were observed in concentrations that induced metamorphosis. Dubilier (1988) hypothesized that enhancement of settlement of *Capitella* sp. I larvae by H<sub>2</sub>S is a sub-lethal toxic effect. However, post-larvae that have been induced by EtOH and MeOH all survived as juveniles and grew in the same rate as those that were obtained by using microbial biofilm, a natural inducer. Organic solvents can therefore be used as a potential artificial inducer of larval metamorphosis in *M. galloprovincialis*. Conversely, Pennington & Hadfield (1989) warned that larval biologists should be aware that contamination of solutions by organic solvents may cause unwanted metamorphosis of larvae.

In conclusion, larvae of *M. galloprovincialis* can be induced to metamorphose

using different chemical compounds such as epinephrine, phenylephrine, clonidine, KCl, NH<sub>4</sub>Cl and organic solvents. These artificial inducers induced normal larval metamorphosis since post-larvae survived and grew at the same rate as those induced by the natural inducer. Exposure time is also an important factor and should be taken into consideration during the screening of artificial inducers. Findings in the present investigation provide additional information on chemical compounds that affect larval metamorphosis of *M. galloprovincialis*.

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## Table Captions

TABLE 1 Chemical compounds used in the present study and their respective manufacturers and concentrations of stock and test solutions. Sigma stands for Sigma Chemical Co. (St Louis, Mo); Wako for Wako Pure Chemical Co. (Osaka, Japan); and –, not investigated.

TABLE 2 Maximum post-larvae (%), EC50 and LC50 values for *M. galloprovincialis* after 72 h in organic solvents in the 24-h exposure assay.

TABLE 3 Growth (in SL) and survival rates of *M. galloprovincialis* post-larvae that metamorphosed using microbial biofilms, EtOH and MeOH.

## Figure Legends

FIGURE 1 Percentages of *M. galloprovincialis* post-larvae in the seven adrenergic agonists at various concentrations in 24-h (a) and continuous (b) exposure assays. Data are means ( $\pm$  SD) of three to 19 replicates.

FIGURE 2 Percentages of *M. galloprovincialis* post-larvae after 72 h in metanephrine (a), VMA (b), and adrenochrome (c) at various concentrations in 24-h and continuous exposure assays. Closed circles ( $\bullet$ ) indicate 24-h exposure assay; open circles ( $\circ$ ), continuous exposure assay. Data are means ( $\pm$  SD) of six to eight replicates.

FIGURE 3 Percentages of *M. galloprovincialis* post-larvae after 72 h in KCl (a) and NH<sub>4</sub>Cl (b) at various concentrations in 24-h and continuous exposure assays. Closed circles ( $\bullet$ ) indicate 24-h exposure assay; open circles ( $\circ$ ), continuous exposure assay. Data are means ( $\pm$  SD) of six replicates.

FIGURE 4 Percentages of *M. galloprovincialis* post-larvae after 72 h in EtOH, MeOH, EG, nPrOH, ACN, DMSO, Ace and hx in 24-h and continuous exposure assays. Closed

circles (●) indicate 24-h exposure assay; open circles (○), continuous exposure assay.

Data are means (+ SD) of three to 21 replicates.

FIGURE 5 Shell growth of *M. galloprovincialis* post-larvae obtained using microbial biofilms, EtOH and MeOH as inducers. Error bars indicate SD (n = 20).

Chemical compound	Manufacturer	Concentration (M)				
		Stock solution	Test solution			
Epinephrine	Sigma (St Louis, Mo)	10 <sup>-3</sup>	10 <sup>-6</sup> , 10 <sup>-5</sup> , 5 × 10 <sup>-5</sup> , 10 <sup>-4</sup>			
Phenylephrine	Wako (Osaka, Japan)	"	"	"	"	"
Clonidine	"	"	"	"	"	"
(-)-3-(3,4-Dihydroxyphenyl)-2-methyl-L-alanine sesquihydrate (α-methyldopa)	Sigma (St Louis, Mo)	"	"	"	"	"
Isoproterenol	"	"	"	"	"	"
Dobutamine	"	"	"	"	"	"
Methoxyphenamine	"	"	"	"	"	"
Metanephrine	Sigma (St Louis, Mo)	10 <sup>-3</sup>	10 <sup>-6</sup> , 10 <sup>-5</sup> , 5 × 10 <sup>-5</sup> , 10 <sup>-4</sup>			
Vanillymandelic acid (VMA)	Wako (Osaka, Japan)	"	"	"	"	"
Adrenochrome	Sigma (St Louis, Mo)	10 <sup>-3</sup>	10 <sup>-6</sup> , 10 <sup>-5</sup> , 5 × 10 <sup>-5</sup> , 10 <sup>-4</sup>			
KCl	Wako (Osaka, Japan)	0.1	10 <sup>-3</sup> , 10 <sup>-2</sup> , 3 × 10 <sup>-2</sup> , 5 × 10 <sup>-2</sup>			
NH <sub>4</sub> Cl	"	"	"	"	"	"
Ethanol (EtOH)	Wako (Osaka, Japan)	5	0.1, 0.5, 0.75, 1.0, 1.5, 2.0, –			
Methanol (MeOH)	"	"	"	"	"	" –
Ethylene glycol (EG)	"	"	"	"	–	" " " –
n-propanol (nPrOH)	"	"	"	"	"	" " " –
Acetonitrile (ACN)	"	"	"	"	–	" – " –
Dimethyl sulfoxide (DMSO)	"	"	"	"	"	" " " – –
Acetone (Ace)	"	"	"	"	"	" " " – –
Hexane (hx)	"	"	"	"	–	" " " – 3.0

TABLE 1

Chemical compound	Maximum post-larvae (%) [concentration (M)]	EC50 (M)	LC50 (M)
Ethanol (EtOH)	70 [0.75]	0.57	1.26
Methanol (MeOH)	67 [1.0]	0.82	2.08
Ethylene glycol (EG)	71 [1.0]	0.79	1.90
n-propanol (nPrOH)	92 [0.1]	0.04	0.30
Acetonitrile (ACN)	78 [0.5]	0.44	1.02
Dimethyl sulfoxide (DMSO)	56 [0.5]	0.49	1.23
Acetone (Ace)	50 [0.5]	0.37	1.22
Hexane (hx)	25 [3.0]	> 100	3.98

TABLE 2

Inducer	Shell growth ( $\mu\text{m/d}$ )		Survival rate (%)
	2 <sup>nd</sup> week	4 <sup>th</sup> week	4 <sup>th</sup> week
Biofilms	41 $\pm$ 23	63 $\pm$ 4	100
EtOH	46 $\pm$ 22	85 $\pm$ 23	100
MeOH	44 $\pm$ 4	66 $\pm$ 32	100

TABLE 3

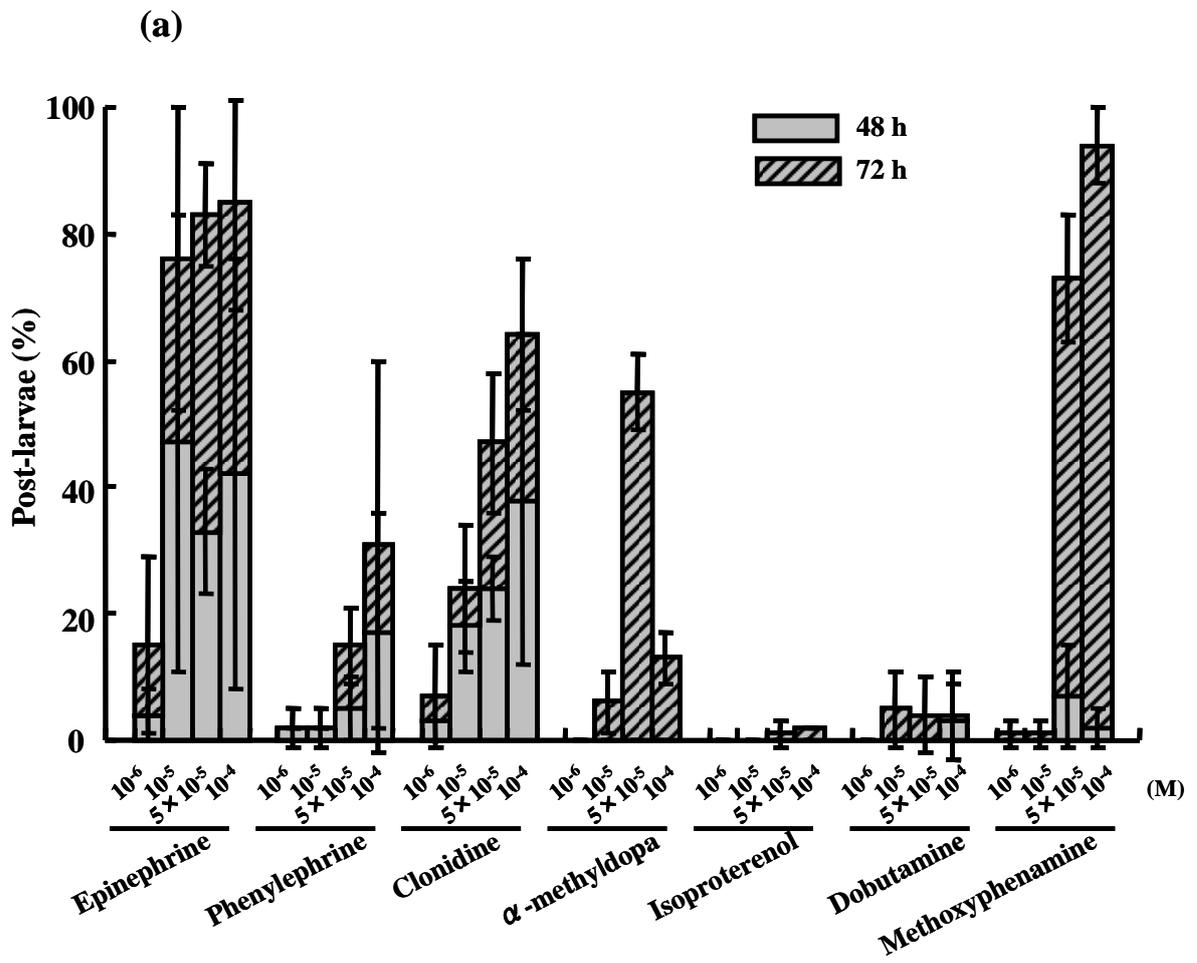


FIGURE 1a

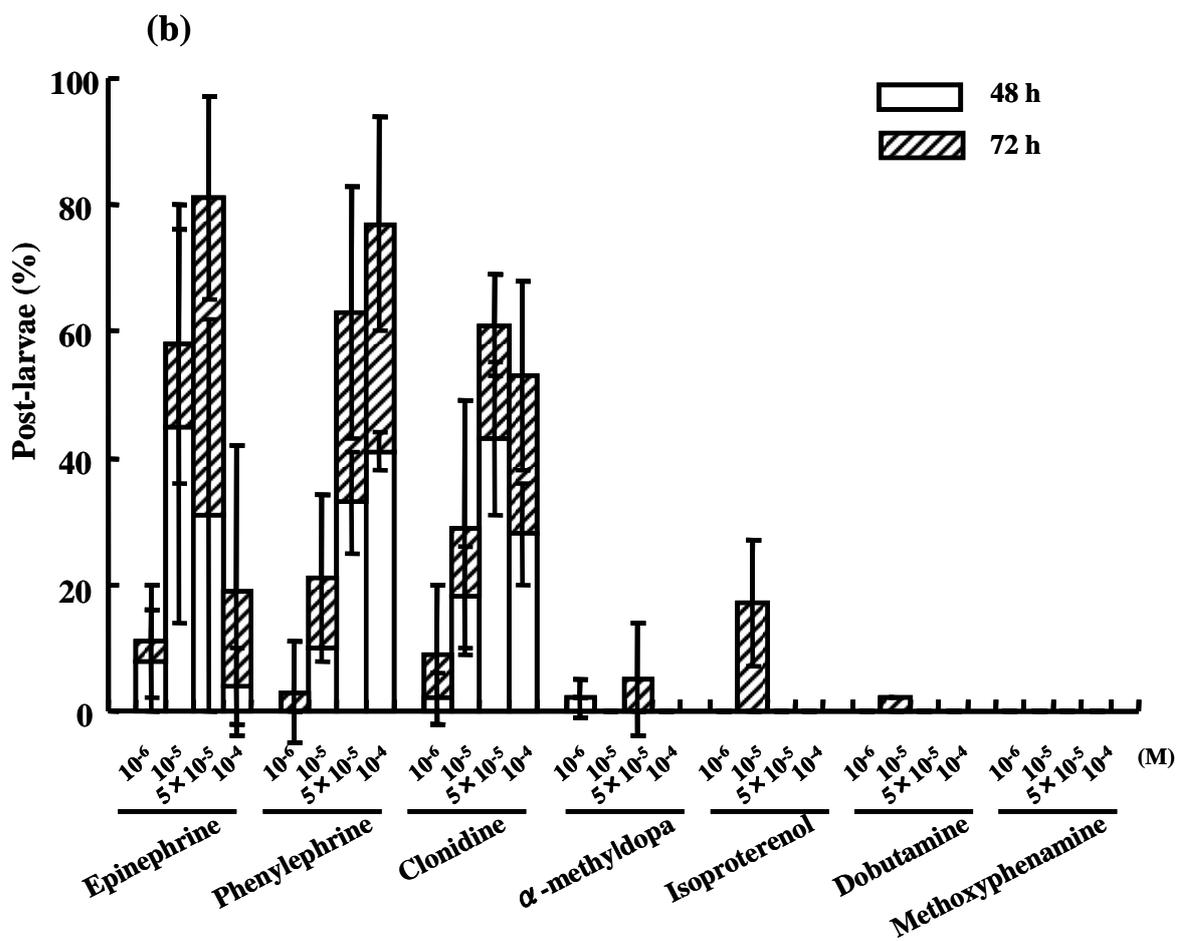


FIGURE 1b

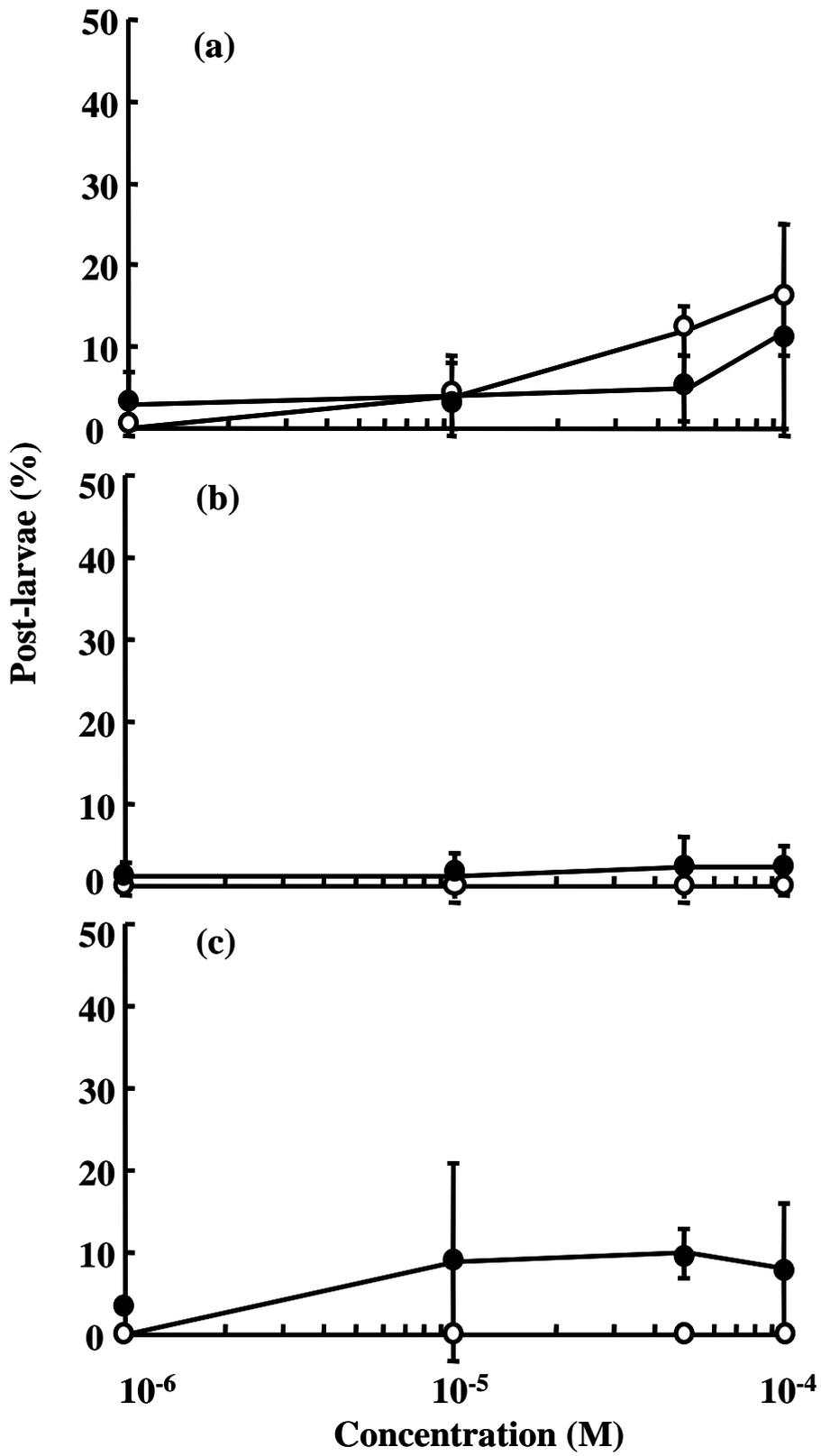


FIGURE 2

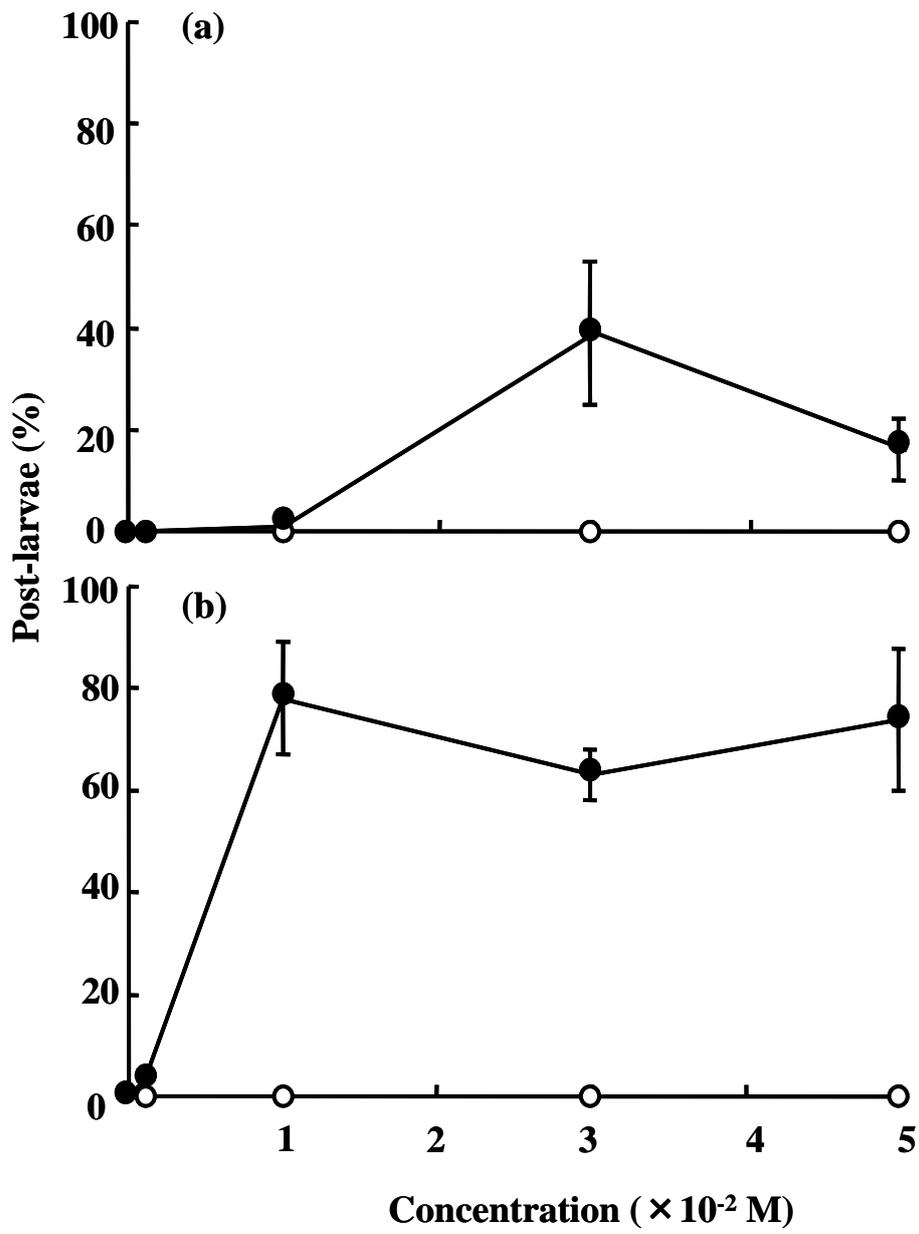


FIGURE 3

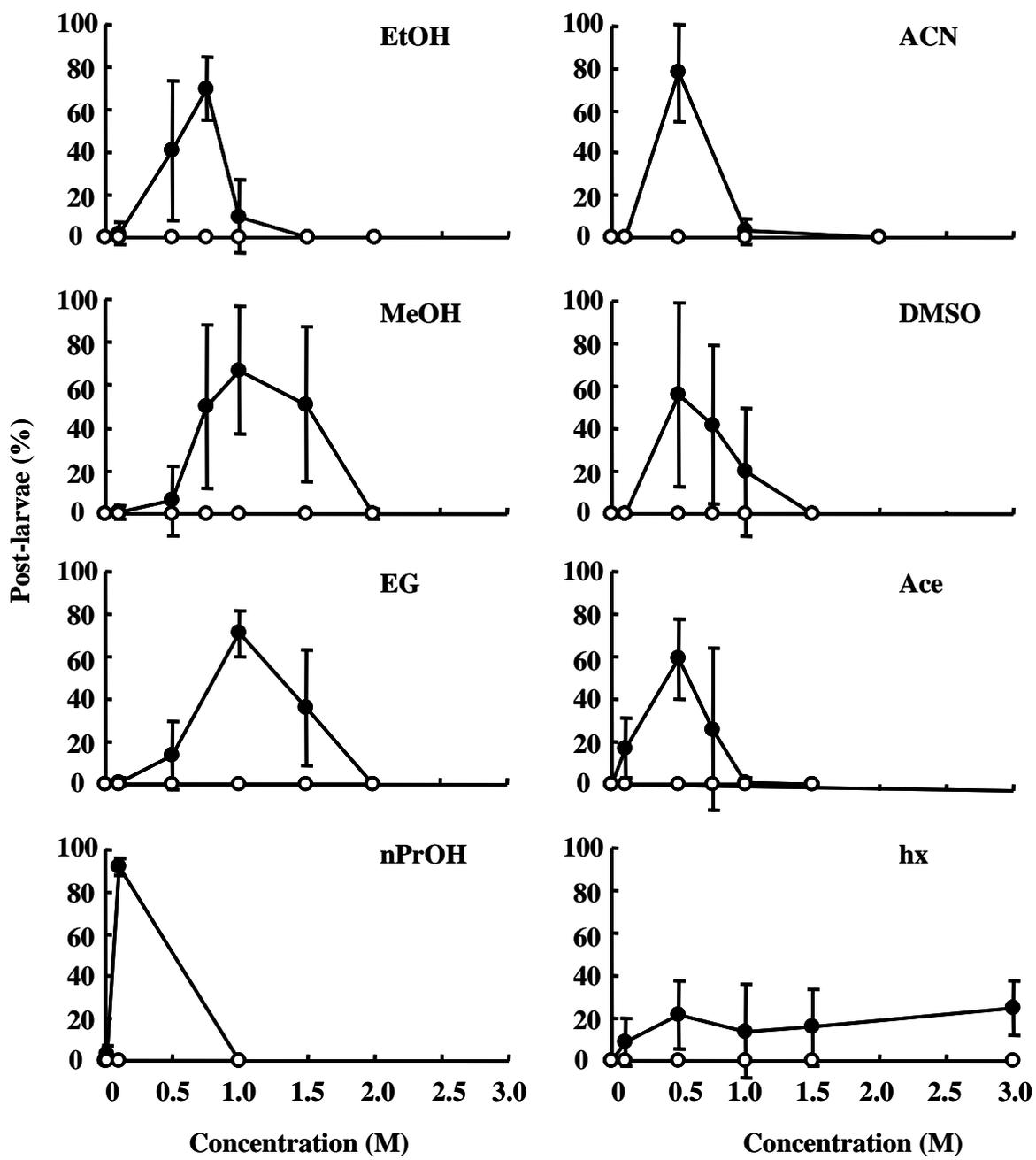


FIGURE 4

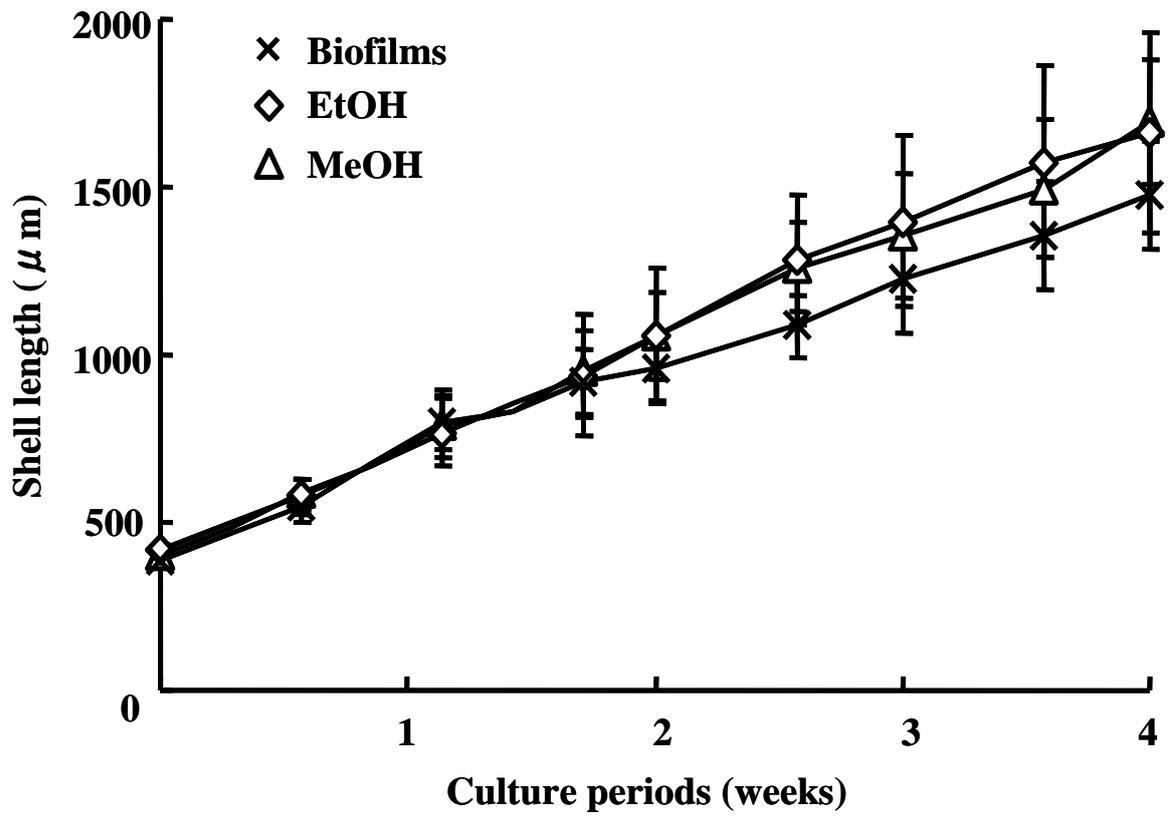


FIGURE 5