Larval Metamorphosis of the Mussel *Mytilus galloprovincialis* Lamarck, 1819 in response to Neurotransmitter Blockers and Tetraethylammonium

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Abstract

Metamorphic response of pediveliger larvae of *Mytilus galloprovincialis* to neurotransmitter blockers and tetratethylammonium chloride (TEA) were investigated through a series of bioassays. The neurotransmitter blockers chlorpromazine, amitriptyline, rauwolscine and idazoxan inhibited *M. galloprovincialis* larval metamorphosis induced by epinephrine. Their respective IC_{50} 's were 4.0×10^{-8} M, 2.6×10^{-5} M, 5.1×10^{-4} M and >100 M. By contrast, atenolol and butoxamine did not inhibit larval metamorphosis. Excess K⁺ ions induced larval metamorphosis at 10^{-3} M to 5×10^{-2} M and 24-h exposure time resulted in maximal metamorphosis. Larval responses to K⁺ were inhibited by TEA at 10^{-3} M. Hence, these compounds can be useful inhibitors of larval metamorphosis for antifouling studies using larvae and juvenile of *M. galloprovincialis*.

Keywords: *Mytilus galloprovincialis*; larval metamorphosis; inhibitor; adrenergic antagonists; tetratethylammonium chloride; antifouling

INTRODUCTION

Mussels are an important aquaculture and fouling species in the world (Kajihara 1985; Hickman 1992; Sakaguchi 2003). As an alien species, the mussel, *Mytilus galloprovincialis* Lamarck, 1819, has been reported to be introduced in the late 1920 (Otani 2002) and is mostly viewed as a macro-fouling organism in Japan because of its colonization on ships' hulls, cooling water systems of power plants and fish cages (Yoo and Kajihara 1983; Sakaguchi and Kajihara 1988; Katsuyama 1995). Previous study suggested that *M. galloprovincialis* accounts for approximate 80% (in biomass) of all fouling organisms on submerged man-made structure in Tokyo Bay (Kajihara 1985). In order to control biofouling including the mussel *M. galloprovincialis*, antifouling (AF) paints and chlorination are widely used in cooling water systems of Japanese electric power plants (Sakaguchi 2003). Efforts are also being made to develop environmentally friendly AF technologies.

M. galloprovincialis larvae have been known to settle and metamorphose in response to bacteria (Satuito et. 1995; Bao et al. 2007a) and macroalgae (Yang et al. 2007). Previous studies also suggested that chemical cues from these natural inducers are involved in larval settlement and metamorphosis (Bao et al. 2007b; Yang et al. 2007).

Despite the evidence that these chemical cues are important for settling larvae in this species, the complete chemical identities of the natural inducer molecules have yet to be identified. Understanding the mussel larval settlement and metamorphosis mechanism is, therefore, important to successfully develop effective AF measures against their colonization of pipes, nets and ship's hulls (Dobretsov and Qian 2003).

In order to understand larval settlement mechanism and find effective AF agents in application in biofouling and AF studies, researchers have switched their focus on the effect of commercially available compounds (e.g. neurotransmitters, neurotransmitter blockers, K⁺ and tetratethylammonium chloride (TEA)) on larval settlement and metamorphosis of many marine invertebrates (Baloun and Morse 1984; Rittschof et al. 1986; Yool et al. 1986; Coon and Bonar 1987; Pawlik 1990; Yamamoto et al. 1998; Satuito et al. 1999; Rittschof et al. 2003). Satuito et al. (1999) showed that M. galloprovincialis larvae can be induced to metamorphose by epinephrine, an adrenergic agonist. By contrast, Yamamoto et al. (1996) reported that epinephrine had no effect on larval settlement and metamorphosis of the barnacle Balanus amphitrite. Coon and Bonar (1987) demonstrated that WB4101, a vertebrate-type alpha₁ adrenergic antagonist can block larval metamorphosis of the Pacific oyster Crassostrea gigas induced by epinephrine. In contrast, Satuito et al. (1999) reported that the adrenergic

antagonist WB4101 had no inhibitive effect on *M. galloprovincialis* larval metamorphosis but instead exhibited agonist activity and induced high percentage of larval metamorphosis. In the case of K^+ , the result of Eyster & Pechenik (1987) showed that excess K^+ ions failed to induce larval metamorphosis in *M. edulis* although K^+ has been known to induce larval metamorphosis of other marine invertebrates (Carpizo-Ituarte & Hadfield 1998; Martinez et al. 1999; Zhao et at. 2003). Baloun and Morse (1984) showed that in *Haliotis rufescens*, induction of larval metamorphosis by excess K^+ ions can be blocked by TEA. However, Yool et al. (1986) and Pawlik (1990) demonstrated that both larvae of *Phestilla sibogae* and *Phragmatopoma californica*, were insensitive to TEA. It is apparent from these studies that different species of marine invertebrates exhibited varying response to chemical compounds.

To obtain information on *M. galloprovincialis* larval response to various chemical compounds and consequently understand the mechanism of its larval metamorphosis, the authors have been investigating the effects of artificial chemical compounds on *M. galloprovincialis* larval metamorphosis. Recently, the authors have demonstrated that vertebrate-type alpha and beta adrenergic agonists and potassium chloride successfully induce larval metamorphosis of *M. galloprovincialis* (Yang et al. 2008). In the present study, the authors investigated the effects of different neurotransmitter blockers and

TEA on successfully larval metamorphosis. The purpose was to identify potential inhibitors for laboratory bioassays in biofouling and AF research.

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 and larval culture in the laboratory were conducted following the procedure described by Yang et al. (2008). 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 filtered sea water (Whatman glass-fiber filter, GF/C: 1.2 μm, 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 μ 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 in 2 l glass beakers at an initial stocking density of 5 larvae ml⁻¹. Larvae were fed a diet of *Chaetoceros gracilis* at 5 × 10^4 cells ml⁻¹day⁻¹. Culture water was changed every other day and the temperature maintained at $17 \pm 1^{\circ}$ 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 µm and >309 µm, respectively (Satuito et al. 2005).

In order to ensure the supply of pediveligers almost all year round, straight-hinge veliger larvae were 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. Conditions for storing and culturing the refrigerated larvae were the same as that reported by Satuito et al. (2005). Refrigeration had no adverse effects on the survival, growth, settlement and metamorphosis of larvae (Satuito et al. 2005).

Chemical compounds

In the present investigation, various chemical compounds used, together with the respective manufacturers and places of purchase, are shown in Table 1. Chemicals selected for the bioassays are known to effectively induce or inhibit larval metamorphosis of other marine invertebrates. Stock solutions of KCl were prepared by diluting these chemicals in 0.22 μ m Millipore filtered seawater (0.22 μ m FSW). Stock solutions of tetratethylammonium chloride (TEA), the six adrenergic antagonists and epinephrine were prepared by dissolving these chemicals initially in distilled water or 0.3 to 0.5 ml of diluted HCl and then diluting it in 0.22 μ m FSW (pH 7.4 to 8.3). Test solutions assayed were prepared by diluting stock solutions in 0.22 μ 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

The inhibitive effects of six vertebrate adrenergic antagonists on larval metamorphosis were investigated following the methods of Coon and Bonar (1987) and Satuito et al. (1999). Twenty larvae were exposed to test solutions of different concentrations of these antagonists for 15 min. Epinephrine, a highly active inducer (Satuito et al. 1999; Yang et al. 2008), was then added into test solutions described above for a final concentration of 10^{-4} M. After three hours exposure time to mix solutions containing the antagonists and 10^{-4} M epinephrine, larvae were rinsed for three times by sequently transferring them containing 0.22 µm FSW and finally transferred to glass Petri dishes (Ø 64 mm × 19 mm height) containing 20 ml of 0.22 µm FSW each. A group of larvae were exposed only to 10^{-4} M epinephrine for three hours and this was viewed as a positive control in the assay. In addition, larvae were also exposed to the six vertebrate antagonists without the addition of 10^{-4} M epinephrine, in order to check for any effect including toxicity of the compounds during the 24-h exposure time.

To investigate the inductive effect of KCl on larval metamorphosis, twenty pediveliger larvae were released in each glass Petri dish containing 20 ml of the test solution. Larvae were subjected to the test solution either in a 24-h exposure bath (Coon and Bonar 1987; Satuito et al. 1999) or in continuous exposure throughout the experimental period. Larvae that were given the 24-h exposure to the test solution were rinsed three times with 0.22 μ m FSW and then finally transferred to Petri dishes containing each of 20 ml of 0.22 μ m FSW. Pediveliger larvae were also exposed to the KCl test solutions for 1, 3 and 24 h. Treated pediveliger larvae were rinsed three times with 0.22 μ m FSW and then finally transferred to Petri dishes containing each of 20 ml of 0.22 μ m FSW.

The inhibiting effect of TEA on larval metamorphosis by excess potassium ions was investigated at different concentrations. Pediveliger larvae were exposed to test solutions of TEA of different concentrations. After 15 min exposure to TEA, 0.1 M KCl was added into the test solutions described above. After the 24-h exposure time to the mix solutions containing TEA and KCl, larvae were rinsed for three times and transferred to Petri dishes each of 20 ml of 0.22 μ m FSW. Larvae were also exposed to TEA without the addition of excess potassium ions, in order to check for any effect including toxicity of TEA during the 24-h exposure time.

In all assays, larvae were checked after 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 nine replicate assays from two to three different culture batches were performed. The exposure time assay using excess potassium ions was conducted in only three replicates from a single culture batch of larvae. A Petri dish containing 20 larvae and 20 ml of 0.22 μ m FSW was always set in each assay as the control. All assays were conducted at $17 \pm 1^{\circ}$ C in a dark environment.

Data analysis

Metamorphosis inducing activities of the chemical compounds were expressed as percentages of post-larvae. The inducing activity of the potassium ion was assessed using the Kruskal-Wallis Test. The inhibitive activities of adrenergic antagonists and TEA were assessed by one-way Analysis of Variance (ANOVA) followed by the Tukey-Kramer Honestly Significant Difference (HSD) test. The data expressed in percentages were arcsine-transformed prior to analysis with one-way ANOVA and Tukey-Kramer HSD test. Values of IC₅₀ of adrenergic antagonists were calculated by probit analysis (Finney, 1971). All statistical computations were performed using the JMPTM software. Differences were considered significant at P < 0.05.

RESULTS

Throughout all experiments, no post-larval metamorphosis of *M. galloprovincialis* was observed in the controls.

Effects of adrenergic antagonists on larval metamorphosis

The effects of the six adrenergic antagonists on larval metamorphosis in the presence and absence of 10^{-4} M epinephrine are as shown in Figure 1. Chlorpromazine (ANOVA: P < 0.001) and amitriptyline (P < 0.001) significantly inhibited larval metamorphosis and the percentages of post-larvae decreased to $32 \pm 15\%$ and $7 \pm 5\%$, respectively. Rauwolscine (P < 0.01) and idazoxan (P < 0.01) also showed inhibiting effects but >50% of larvae still metamorphosed to post-larvae in both cases. By contrast, the other antagonist atenolol (P > 0.05) and butoxamine (P > 0.05) did not exhibit any inhibitive effects on metamorphosis at all concentrations tested. Furthermore, rauwolscine and amitriptyline exhibited agonist activity at 10^{-4} M and induced larval metamorphosis even in the absence of epinephrine. larval mortality of $1 \pm 2\%$ was observed only in 10^{-6} M rauwolscine. Based on the results shown in Figure 1, the concentrations that inhibited 50% larval metamorphosis (IC₅₀) for the six adrenergic antagonists were calculated as shown in Table 2. IC₅₀ of chlorpromazine was 4.0×10^{-8} M, and this was 600-times more effective than amitriptyline. The IC₅₀s of rauwolscine and idazoxan were 5.1×10^{-4} M and >100 M, respectively. By contrast, atenolol and butoxamine did not inhibit larval metamorphosis.

The inductive effect of KCl on larval metamorphosis

Various concentrations and exposure times were investigated to obtain maximal results for KCl, an inducer of *M. galloprovincialis* larval metamorphosis. In the continuous exposure assay, no larvae were observed to metamorphose in KCl (P=1) after 72 h. The percentages of post-larvae in KCl after 72 h in the 24-h exposure assay are as shown in Figure 2. Potassium chloride exhibited significantly inducing activities (Kruskal-wallis test: P < 0.001) after 72 h in the 24-h exposure assay and induced maximum post-larval metamorphosis at 3 × 10⁻² M (Figure 2). No larval mortality was observed in the 24-h and continuous exposure assay. The percentages of post-larval metamorphosis in 3×10^{-2} M KCl at various exposure periods are as shown in Figure 3. The percentages of post-larval metamorphosis in KCl increased with increasing exposure time up to 24 h and maximum post-larval metamorphosis was observed after larvae were exposed for 24 h. No larval mortality was observed throughout the assays.

The effect of tetraethylammonium chloride (TEA) on larval metamorphosis

The effects of TEA, a potassium channel blocker, on the percentages of post-larval metamorphosis with and without excess potassium ions are shown in Figure 4. When larvae were exposed to 10^{-4} M TEA for 24 h, the percentage of larval metamorphosis did not significantly decrease (ANOVA: P > 0.05, Figure 4). One millimolar TEA significantly inhibited larval metamorphosis even in the presence of KCl (ANOVA: P < 0.05, Figure 4). No mortality was observed at all concentrations of TEA tested in the case when potassium ions were not in excess condition. Mortalities were < 5% when larvae were exposed to 10^{-4} M TEA in the presence of KCl.

DISCUSSION

Numerous literature have documented different pharmacological compounds that induce or inhibit larval metamorphosis of many marine invertebrates including hydroids, bryozoans, polychaetes, mollusks, barnacles, sea urchins and ascidians. These studies provide valuable information that has application in the fields of biofouling, antifouling and aquaculture (Cooper 1983; Rittschof et al. 1992; Martinez et al. 1999; Zhao et al. 2003; Gapasin and Polohan 2004; García-Lavandeira et al. 2005; Yu et al. 2007; Zhou et al. 2009). In the present investigation, the authors have demonstrated that chemical compounds chlorpromazine, amitriptyline and TEA inhibit larval metamorphosis of the mussel *M. galloprovincialis*.

Previous studies have demonstrated that phentolamine, a vertebrate-type alpha adrenergic antagonist, can inhibit *M. galloprovincialis* larval metamorphosis (Yamamoto et al. 1998; Satuito et al. 1999; 2005). It indicates that vertebrate-type alpha receptor may be involved in larval metamorphosis in this species. Mediation of the more selective alpha₁ and alpha₂ receptor remains unclear. In our previous study, we

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have demonstrated that larvae of the mussel M. galloprovincialis can be induced to metamorphose using neuroactive compounds including vertebrate-type alpha and beta adrenergic agonist phenylephrine, clonidine, α -methyldopa and methoxyphenamine (Yang et al. 2008). In the present investigation, larval metamorphosis of M. galloprovincialis induced by epinephrine was inhibited by vertebrate-type alpha₁ adrenergic antagonist chlorpromazine and amitriptyline, implying that the vertebrate-type alpha₁ adrenoceptor may mediate larval metamorphosis of this species. Chlorpromazine and amitriptyline also inhibited larval metamorphosis in the mollusks (Coon and Bonar 1987; Yamamoto et al. 1998) and a barnacle species (Yamamoto et al. 1996; 1998). In addition, the vertebrate-type beta adrenergic antagonist atenolol and butoxamine exhibited no inhibiting activities in the present assays. Previous report also demonstrated that other vertebrate-type beta adrenergic antagonists including propranolol and timolol had no inhibiting effect in this species (Satuito et al. 1999) These present investigation suggests that larval metamorphosis of *M. galloprovincialis* can be effectively controlled by adrenergic agonists and antagonists.

Potassium ion is known to induce larval metamorphosis of many marine invertebrates. In the present study, excess potassium induced *M. galloprovincialis* larval metamorphosis and 24-h exposure time was needed to obtain maximal results. This result is consistent with our previous study (Yang et al. 2008). Interestingly, Eyster & Pechenik (1987) reported that excess K^+ ions can not induce *M. edulis* larval metamorphosis although they both belong to the *Mytilus*. This inconsistency indicates that the effect of K^+ on *Mytilus* larval metamorphosis showed high species specificity.

The present investigation has also demonstrated that *M. galloprovincialis* larval metamorphosis induced by KCl can be inhibited by TEA, a potassium-channel blocker. The mechanism by which potassium ion induces larval metamorphosis of many marine invertebrate has been hypothesized in literatures (Baloun and Morse 1984; Yool et al. 1986; Todd et al. 1991; Carpizo-Ituarte and Hadfield 1998; Hadfield et al. 2000; Zhao et al. 2003). For example, potassium acts on larval nervous systems, producing a generalized activation (Todd et al. 1991), or that K⁺ acts directly on responding target issues (Yool et al. 1986; Pechenik et al. 1995). Hadfield et al. (2000) demonstrated that potassium does not act only the metamorphic-signal receptor cells, but at other sites downstream in the metamorphic signal transduction pathway. However, many researchers agree with a hypothesis that excess K⁺ ions act directly depolarizing excitable cells involved in the larval perception of inductive stimuli (Baloun and Morse 1984; Yool et al. 1986; Martinez et al. 1999; Zhao et al. 2003; Yu et al. 2007).

In conclusion, larval metamorphosis of M. galloprovincialis can be inhibited by

TEA and neurotransmitter blockers such as chlorpromazine and amitriptyline. The present study provides additional information on pharmacological compounds that inhibit metamorphosis of larvae of the mussel *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 = Sigma Chemical Co. (St Louis, Mo); Wako = Wako Pure Chemical Co. (Osaka, Japan); - = not investigated.

TABLE 2 IC₅₀s values for *M. galloprovincialis* after 72 h in adrenergic antagonists. N= no activity at all concentrations tested.

Figure Legends

FIGURE 1 Percentages of *M. galloprovincialis* post-larvae after 72 h obtained by exposure to different concentrations of the six adrenergic antagonists, in the absence (open boxes) or presence (shaded boxes) of 10^{-4} M epinephrine. Data are means (+ SD) of 6 to 9 replicates.

FIGURE 2 Percentages of *M. galloprovincialis* post-larvae after 72 h in excess potassium ions at various concentrations in the 24-h exposure assay. Data are means (\pm SD) of 6 to 9 replicates.

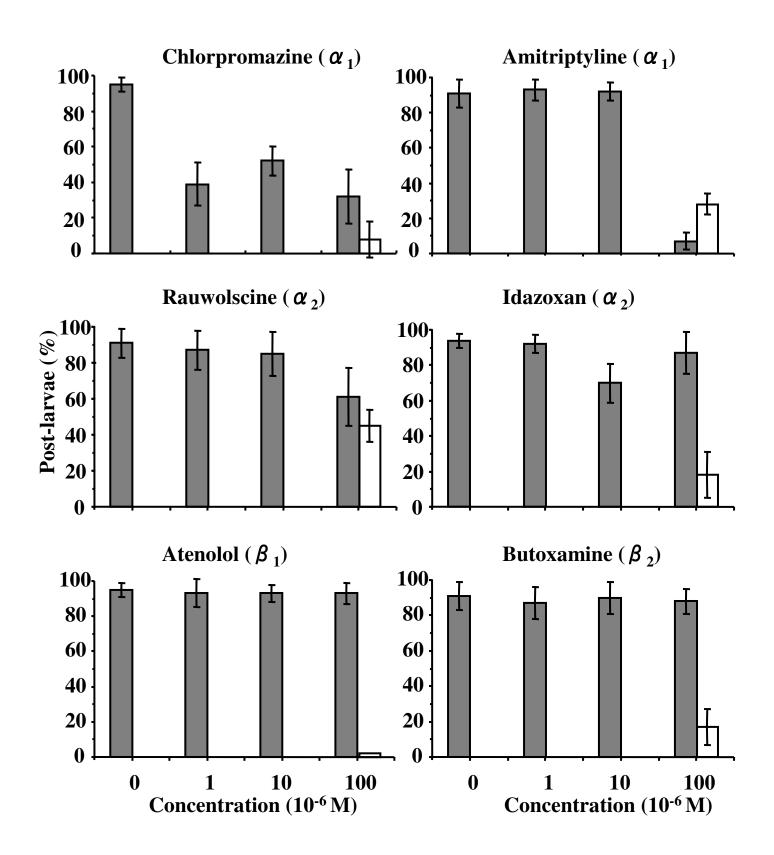
FIGURE 3 Percentages of *M. galloprovincialis* post-larvae after 72 h obtained by different exposure time to 3×10^{-2} M KCl. Data are means (± SD) of 3 replicates.

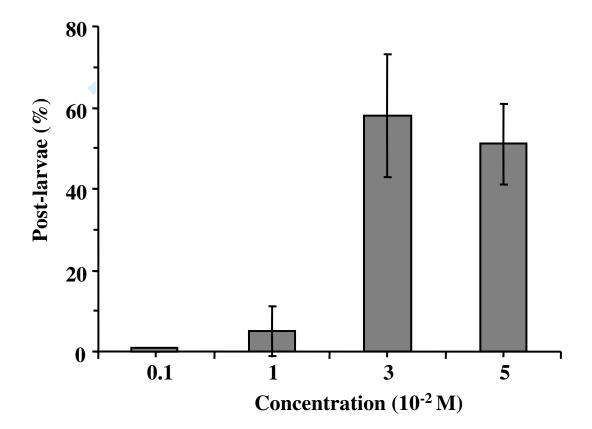
FIGURE 4 Percentages of *M. galloprovincialis* post-larvae after 72 h obtained by exposure to different concentrations of tetraethylammonium (TEA), in the absence (open boxes) or presence (shaded boxes) of 3×10^{-2} M KCl. Data are means (± SD) of 6 replicates.

Chemical compound	Manufacturer	Concentration (M)	
		Stock solution	Test solution
Chlorpromazine	Sigma (St Louis, Mo)	10-3	10-6 10-5 10-4
Amitriptyline	"	11	<i>II II II</i>
Rauwolscine	"	"	" " "
Idazoxan	"	"	11 11 11
Atenolol	"	11	11 11 11
Butoxamine	"	11	<i>II II II</i>
Epinephrine	"	"	//
KCl	Wako (Osaka, Japan)	0.1	10 ⁻³ 10 ⁻² 3×10 ⁻² 5×10 ⁻²
Tetraethylammonium chloride (TEA)	Sigma (St Louis, Mo)	10-3	10-4 10-3

TABLE 1

Adrenergic antagonist	Selectivity	IC ₅₀ (M)
Chlorpromazine	alpha ₁	4.0×10 ⁻⁸
Amitriptyline	alpha ₁	2.6×10 ⁻⁵
Rauwolscine	alpha ₂	5.1×10 ⁻⁴
Idazoxan	alpha ₂	> 100
Atenolol	beta ₁	Ν
Butoxamine	beta ₂	Ν





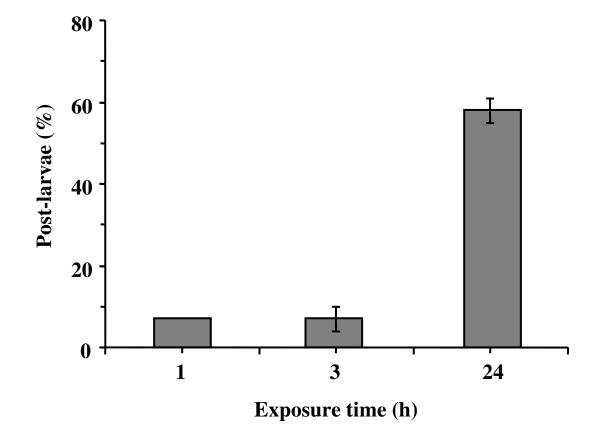


FIGURE 3

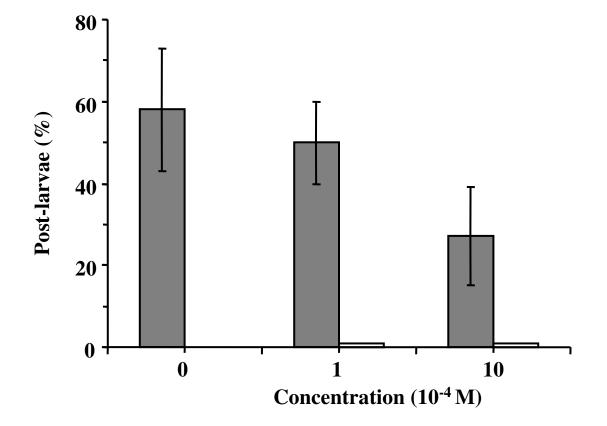


FIGURE 4