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ORIGINAL ARTICLE

Induced metamorphosis in larvae of the veined rapa whelk *Rapana venosa* using chemical cues

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Abstract

The larval metamorphosis of the veined rapa whelk *Rapana venosa* was investigated by exposing competent larvae to the following seven chemical cues at different concentrations and exposure time: EPI (epinephrine), serotonin, L-DOPA (L-3, 4-dihydroxyphenylalanine), GABA (γ-aminobutyric acid), acetylcholine chloride, KCl (potassium chloride), $CaCl_2$ (calcium chloride). Larvae of *R. venosa* which lost their velum after the veliger stage, crawled on their foot and produced an adult shell were considered to have metamorphosed. Compared with the control treatments, there were significantly higher percentages of larval metamorphosis in the following groups: EPI (10^{-3} M) at 6 h, L-DOPA (10^{-3} M, 10^{-4} M) at 6 h, L-DOPA (10^{-5} M) at 24 h, GABA (10^{-5} M) at 24 h, acetylcholine chloride (10^{-4} M) at 6 h, acetylcholine chloride (10^{-4} M, 10^{-6} M, 10^{-7} M) at 24 h and 5 mM $CaCl_2$

et al. 2012). GABA (γ-aminobutyric acid) and KCl stimulated the best attachment and metamorphic responses in the gastropod *Haliotis asinina* (Linnaeus, 1758) postlarvae (Gapasin & Polohan 2004). Dopamine (L-3, 4-dihydroxyphenylalanine) at 5 μM induced a high metamorphosis rate (83%) of the larvae in the sea cucumber *Apostichopus japonicus* (Selenka, 1867) (Matsuura et al. 2009).

The veined rapa whelk *Rapana venosa* (Valenciennes, 1846), native to the coasts of China, Japan and Korea, is one of the most commercially important gastropod species in Asia (Chung et al. 2002). It is also an invasive species in Europe and North America (Chandler et al. 2008). In recent decades, the natural resources of *R. venosa* in China have declined because of over-exploitation, habitat loss, pollution and other factors (Yang et al. 2008a; An et al. 2013; Ban et al. 2014). Consequently, the development of *R. venosa* hatchery techniques has become of economic and ecological interest and it was one of the goals of the National 'Twelfth Five-Year' Plan for Science & Technology Support in China. Nevertheless, progress in the *R. venosa* culture industry is still hampered due to low larval settlement and metamorphosis rates. Artificial farming of this species still mainly relies on the collection of juveniles from the wild. An increase in the percentages of larval settlement and metamorphosis is critical for production of *R. venosa*. However, no studies on larval settlement and metamorphosis of *R. venosa* have been reported. In the present study, the effects of seven chemical cues (EPI, serotonin, L-DOPA, GABA, acetylcholine chloride, KCl, $CaCl_2$) on inducing *R. venosa* metamorphosis were investigated in order to provide a potential solution for the success of large-scale artificial seeding of *R. venosa*.

Materials and methods

Spawning and hatching

Adult *Rapana venosa* with shell height of ≥80 mm were obtained from the wild (Yantai, Shandong Province, China) and acclimated at a shellfish culture farm in Yantai. The veined rapa whelk spawned naturally, after culturing in sand-filtered seawater (SFSW) with 30‰ salinity and a temperature of 22 to 25°C for 20 days. On 7 June 2014, the first batch of planktonic larvae hatched from egg masses.

Larval culture

Approximately 24 h after hatching, the planktonic larvae were transferred into a larval culture tank containing 100 m^3 SFSW. During cultivation, water temperature was maintained at 25 ± 3 °C with salinity at 30 ± 1‰. A mixed diet of the algae *Pseudoisochrysis paradoxa* F.D. Ott, *Chlorella vulgaris* Beyerinck and *Teraselmis chuii* Butcher was provided three times every day. After 1–2 days post-hatching, the planktonic larvae had one spiral whorl and a two-lobed velum; after 3–6 days post-hatching, the planktonic larvae had two spiral whorls and a two-lobed velum. After 7–25 days post-hatching, the planktonic larvae had three spiral whorls and a four-lobed velum. After 26–30 days post-hatching, the planktonic larvae had four spiral whorls and a four-lobed velum. Larvae in this period, whose shell length was 1100 μm to 1200 μm, were selected for the metamorphosis assays.

Metamorphosis cues

The following five neuroactive and two ionic compounds have been reported to have abilities to induce metamorphosis in some marine invertebrates: EPI, serotonin, L-DOPA, GABA, acetylcholine chloride, KCl, $CaCl_2$; they were tested in triplicate against the control groups in our study. The neuroactive compounds were all set at five different concentrations (10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} M) and the two ionic compounds were also used at five different concentrations (5, 10, 15, 20 and 30 mM). All concentrations of the chemical cues were freshly configured to ensure their efficacy.

Metamorphosis assays

Experiments were carried out in Petri dishes (120 mm × 24 mm) containing 100 ml of the test solution and 20 larvae. Three replicates were conducted in each treatment, and the control groups contained 100 ml of 0.45 μm membrane filtered seawater (FSW) without chemical cues and other conditions were the same as the treated groups. During the experiment, no food was provided. The larvae were exposed to different concentrations of test solutions for 6 h and 24 h at 26 ± 2°C with weak light. Larval metamorphosis and mortality were monitored after 6 h and 24 h using a microscope. Larvae which lost their velum, crawled on their foot and produced the adult shell were considered to have metamorphosed. Larval mortality was indicated when a larva did not have any characteristics of living (swimming, crawling or movements of viscera). The metamorphosis rate did not take account of the dead larvae.

Statistical analysis

Percentages of both metamorphosis and mortality were analysed using the one-way ANOVA from

SPSS 16.0. The Least Significant Difference Test (LSD) was carried out between the treatment groups. The results were considered to be significantly different when $P < 0.05$.

Results

Mortality of larvae exposed to different chemical cues

The mortality of the larvae exposed to the seven chemical cues are shown in Figure 1. Larval mortality increased with the chemical cue concentrations and inductive time. The treatment of 10^{-3} M EPI produced 36.67% and 100.00% mortality in *Rapana venosa* larvae after 6 h and 24 h, respectively, which differed significantly from the larval controls ($P < 0.05$). Serotonin was toxic to the metamorphosed larvae, especially at a longer duration (24 h). Larval mortality in the groups using serotonin (10^{-3} M, 10^{-4} M, 10^{-5} M) after 24 h was 100.00%, 100.00% and 90.00 ± 10.00%, respectively ($P < 0.05$). The groups of 10^{-3} M and 10^{-4} M L-DOPA had high larval mortality whether for 6 h or 24 h ($P < 0.05$). There were significant effects on larval mortality after long exposure (24 h) to GABA except for 10^{-7} M (Figure 1D). The mortality induced by 10^{-3} M and 10^{-4} M GABA was 100.00% for both groups after exposure for 24 h ($P < 0.05$). Acetylcholine chloride and KCl had a similar effect on *R. venosa* larval mortality (Figure 1E, F). The percentages of mortality induced by 10^{-3} M acetylcholine chloride and 30 mM KCl after 24 h were 93.33 ± 5.77% and 96.67 ± 5.77%, which differed significantly from the larval controls ($P < 0.05$). $CaCl_2$ did not significantly affect *R. venosa* larval mortality, compared with the control groups ($P > 0.05$).

Metamorphosis of larvae exposed to different chemical cues

The results of the metamorphosis rate of *Rapana venosa* obtained for the seven chemical treatments after 6 h and 24 h are shown in Figure 2. Compared with the control treatments, there were significantly higher percentages of larval metamorphosis in the groups of EPI (10^{-3} M) at 6 h, L-DOPA (10^{-3} M, 10^{-4} M) at 6 h, L-DOPA (10^{-5} M) at 24 h, GABA (10^{-5} M) at 24 h, acetylcholine chloride (10^{-4} M) at 6 h, acetylcholine chloride (10^{-4} M, 10^{-6} M, 10^{-7} M) at 24 h and 5 mM $CaCl_2$ at 24 h ($P < 0.05$).

EPI had little effect in inducing larval metamorphosis. Though the percentage of larval metamorphosis induced by 10^{-3} M at 6 h was 50.00 ± 10.00%, which was significantly higher than the control groups, EPI at other concentrations did not have any significant difference compared with the control groups ($P > 0.05$).

Serotonin and KCl had no inductive effect on larval metamorphosis since these treatments did not induce any significant difference compared with the control groups ($P > 0.05$).

As shown in Figure 2C, L-DOPA had significant inductive effects on larval metamorphosis. The highest percentage of larval metamorphosis was induced by 10^{-4} M of L-DOPA at 6 h, having the value of 60.00 ± 10.00%, which was significantly higher than the control groups ($P < 0.05$). The metamorphosis percentages of the larvae exposed to L-DOPA (10^{-3} M) at 6 h and L-DOPA (10^{-5} M) at 24 h were 36.67 ± 11.55% and 30.00 ± 10.00%, respectively. They were also significantly higher than those recorded in the control groups ($P < 0.05$).

GABA had little effect in inducing larval metamorphosis. Compared with the control treatments, only at 24 h did GABA (10^{-5} M) significantly induce larval metamorphosis ($P < 0.05$).

Acetylcholine chloride had significant effects in inducing larval metamorphosis. The significantly higher percentages of larval metamorphosis induced by acetylcholine chloride were observed at 10^{-4} M after 6 h, and 10^{-4} M, 10^{-6} M, 10^{-7} M after 24 h, having the values of 13.33 ± 5.77%, 20.00 ± 10.00%, 26.67 ± 5.77%, and 33.33 ± 7.64% respectively, compared with the results of the control groups ($P < 0.05$).

$CaCl_2$ had little effect inducing larval metamorphosis. $CaCl_2$ failed to induce metamorphosis of *R. venosa* larvae, except for 5 mM concentrations after 24 h ($P < 0.05$).

The rates including both mortality and metamorphosis are indicated in Table I. High larval mortalities were detected in the groups of EPI (10^{-3} M for 6 h), L-DOPA (10^{-3} M, 10^{-4} M for 6 h) and GABA (10^{-5} M for 24 h), although they induced high larval metamorphosis. In contrast, the groups of L-DOPA (10^{-5} M for 24 h), acetylcholine chloride (10^{-4} M for 6 h; 10^{-4} M, 10^{-6} M, 10^{-7} M for 24 h) and $CaCl_2$ (5 mM for 24 h) could induce high larval metamorphosis with low mortality. Acetylcholine chloride and $CaCl_2$ were indicated as active inducers with low toxicity among the seven cues.

Discussion

Metamorphosis is an irreversible process including loss of the larval feeding organ, development of the gills and production of the adult shell (García-Lavandeira et al. 2005). In the *Rapana venosa* culture industry, there is the serious problem that few competent larvae can successfully complete

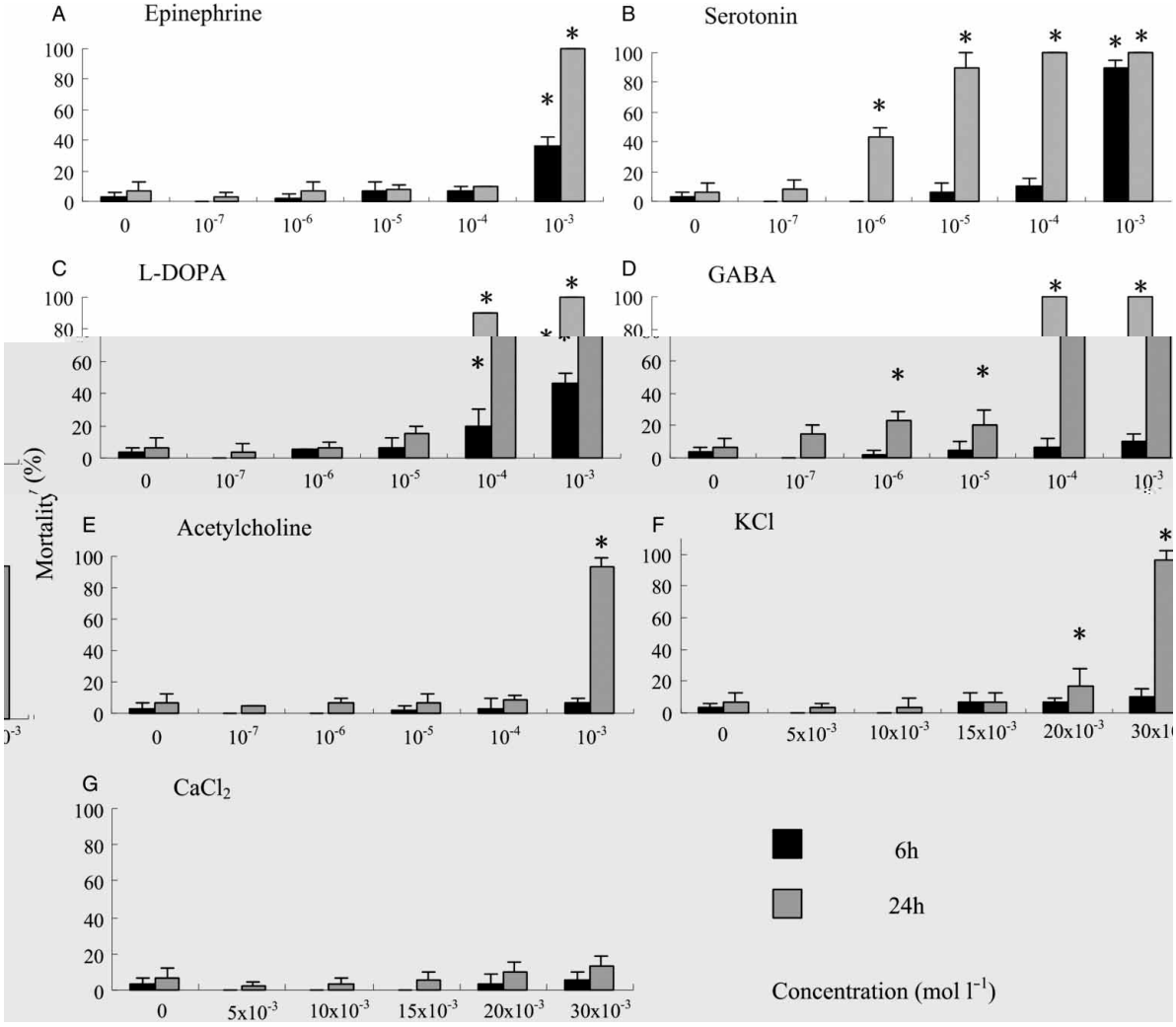

Figure 1. Mortality of competent larvae of *Rapana venosa* exposed to various concentrations of different chemical cues for 6 h and 24 h. (A) epinephrine, (B) serotonin, (C) L-DOPA, (D) GABA, (E) acetylcholine, (F) KCl, (G) $CaCl_2$.
Asterisks indicate significant levels at 95% ($P < 0.05$).

metamorphosis and settlement under the conditions of artificial seeding, which was also found in the control groups of our study. A series of studies have reported that some neuroactive and ionic compounds can effectively induce larval metamorphosis (Beiras & Widdows 1995; Biggers & Laufer 1999; Bao et al. 2007). Neuronal and neuroendocrine activities are thought to control the metamorphosis process (García-Lavandeira et al. 2005). In this study, it is demonstrated that the larvae of *R. venosa* can be induced to metamorphose by exposure to EPI, L-DOPA, GABA, acetylcholine and $CaCl_2$. The findings support previous reports that chemical cues have abilities to induce metamorphosis in marine invertebrates.

EPI, a type of catecholamine, is one of the most common and useful inducing substances. There is a lot of evidence that EPI is involved in the process of settlement and metamorphosis. For example, the level of EPI increases as the larvae grow and reaches a maximum before metamorphosis (Coon & Bonar 1986). EPI has been proved to be an active inducer of metamorphosis in *Mytilus galloprovincialis* (Lamarck, 1819) and *Venerupis corrugata* (Gmelin, 1791) at 10^{-4} M, 10^{-5} M and 10^{-6} M (García-Lavandeira et al. 2005), while our experiments show that EPI had the ability to induce metamorphosis of *R. venosa* larvae only at 10^{-3} M for 6 h but also resulted in a high mortality.

Serotonin, a derivative of tryptophan, can induce metamorphosis of some marine invertebrates. For example, the larvae of the mud snail *Ilyanassa obsoleta* (Say, 1822) and *Pinctada maxima* (Jameson, 1901) can effectively be induced by serotonin (Levantine & Bonar 1986; Zhao et al. 2003). In this study, serotonin did not induce any significant larval metamorphosis of *R. venosa* and resulted in high mortality, which is consistent with previous studies in which

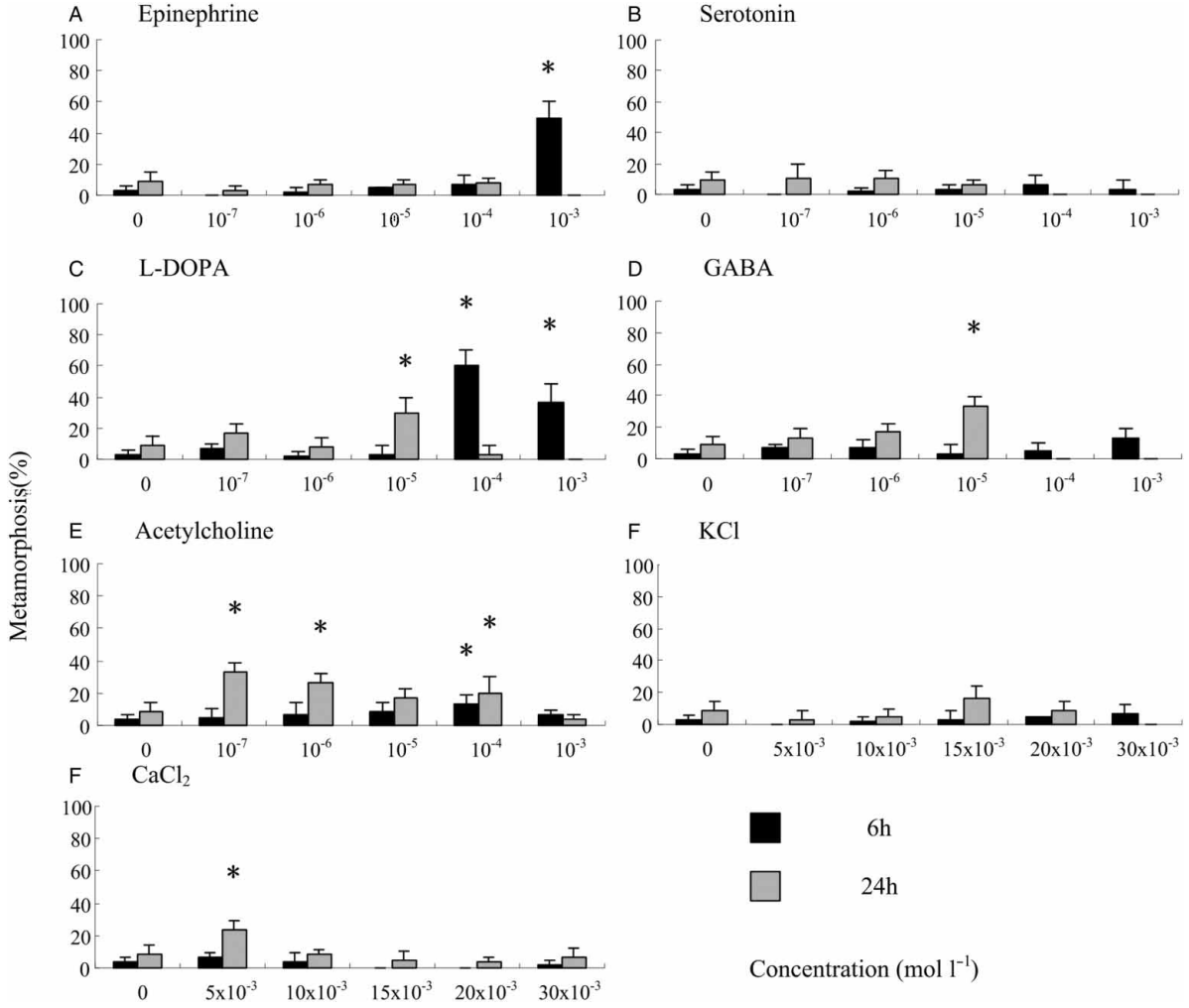

Figure 2. Metamorphosis percentages of competent larvae of *Rapana venosa* exposed to various concentrations of different chemical cues for 6 h and 24 h. (A) epinephrine, (B) serotonin, (C) L-DOPA, (D) GABA, (E) acetylcholine, (F) KCl, (G) $CaCl_2$. Asterisks indicate significant levels at 95% ($P < 0.05$).

some deleterious effects on *Apostichopus japonicus* were observed at higher concentrations of serotonin (Sun et al. 2014).

L-DOPA, a derivative of tyrosine, is thought to induce the larvae indirectly by being absorbed first and then changed into dopamine (Bonar et al. 1990). Some studies found that L-DOPA could actively induce settlement and metamorphosis after a specific time (Pawlik 1990; Pires & Hadfield 1991). In the present study, 10^{-3} M and 10^{-4} M L-DOPA can significantly induce higher percentages of metamorphosis in *R. venosa* larvae at 6 h than that at 24 h. It is suggested that L-DOPA at 10^{-3} M and 10^{-4} M was toxic to *R. venosa* after 24 h, and similar results were observed for *Pinctada martensii* (Dunker, 1873) (Yu et al. 2008).

GABA, an amino acid derivative and a neurotransmitter, has been found to exist in gastropod mucus (Laimek et al. 2008). Morse et al. (1979) first confirmed that GABA had an inducing effect on invertebrate larval metamorphosis in the gastropod *Haliotis rufescens* (Swainson, 1822). GABA was identified as an active inducer of metamorphosis in *Ostrea edulis* (Linnaeus, 1758), which induced a high percentage of metamorphosis (60%) with 10^{-4} M GABA (García-Lavandeira et al. 2005). Although the percentage of larval metamorphosis induced by GABA was significantly higher only at 10^{-5} M after 24 h, the present results still demonstrate the inducing effects of GABA in *R. venosa*.

Acetylcholine, an ester of acetic acid and choline, plays an important role in bridging neuron–neuron and neuron–muscular synapses in marine invertebrates (Zhao et al. 2003). Acetylcholine can induce the contraction of the gill filaments in *Mytilus edulis* (Linnaeus, 1758) (Beiras & Widdows 1995). So far, research on acetylcholine has been mainly on the applications to larval settlement, while reports on

Table I. Rates of mortality and metamorphosis of *Rapana venosa* larvae in response to seven chemical cues. Values marked with an asterisk (*) are significantly different from the control groups ($P < 0.05$).

Chemical cues	Mean rates (%)	0 M	10^{-7} M	10^{-6} M	10^{-5} M	10^{-4} M	10^{-3} M
EPI	*6 h Mortality*	3.33	0	1.67	6.67	6.67	36.67*
	6 h Metamorphosis	3.33	0	1.67	5	6.67	50*
	24 h Mortality	6.67	3.33	6.67	8.33	10	100*
	24 h Metamorphosis	8.83	3.33	6.67	6.67	8.33	0
Serotonin	*6 h Mortality*	3.33	0	0	6.67	10	90*
	6 h Metamorphosis	3.33	0	1.67	3.33	6.67	3.33
	24 h Mortality	6.67	8.33	43.33*	90*	100*	100*
	24 h Metamorphosis	8.83	10	10	6.67	0	0
L-DOPA	*6 h Mortality*	3.33	0	5	6.67	20*	46.67*
	6 h Metamorphosis	3.33	6.67	1.67	3.33	60*	36.67*
	24 h Mortality	6.67	3.33	6.67	15	90*	100*
	24 h Metamorphosis	8.83	16.67	8.33	30*	3.33	0
GABA	*6 h Mortality*	3.33	0	1.67	5	6.67	10
	6 h Metamorphosis	3.33	6.67	6.67	3.33	5	13.33
	24 h Mortality	6.67	15	23.33*	20*	100*	100*
	24 h Metamorphosis	8.83	13.33	16.67	33.33*	0	0
Acetylcholine	*6 h Mortality*	3.33	0	0	1.67	3.33	6.67
	6 h Metamorphosis	3.33	5	6.67	8.33	13.33*	6.67
	24 h Mortality	6.67	5	6.67	6.67	8.33	93.33*
	24 h Metamorphosis	8.83	33.33*	26.67*	16.67	20*	3.33
KCl	*6 h Mortality*	3.33	0	0	6.67	6.67	10
	6 h Metamorphosis	3.33	0	1.67	3.33	5	6.67
	24 h Mortality	6.67	3.33	3.33	6.67	16.67*	96.67*
	24 h Metamorphosis	8.83	3.33	5	16.67	8.33	0
$CaCl_2$	*6 h Mortality*	3.33	0	0	0	3.33	5
	6 h Metamorphosis	3.33	6.67	3.33	0	0	1.67
	24 h Mortality	6.67	1.67	3.33	5	10	13.33
	24 h Metamorphosis	8.83	23.33*	8.33	5	3.33	6.67

acetylcholine and its implications for larval metamorphosis are few. In this study, acetylcholine chloride was an active inducer of *R. venosa* larval metamorphosis, which is consistent with the results in *Crassostrea gigas* (Thunberg, 1793) reported by Beiras and Widdows (1995). Acetylcholine chloride induced high percentages of larval metamorphosis and had low mortality except at 10^{-3} M. Acetylcholine did not significantly induce a higher metamorphosis rate at the concentration of 10^{-5} M for 24 h while it was efficient at the concentrations of 10^{-7} M, 10^{-6} M and 10^{-4} M. Sensitivities of *R. venosa* larvae to different concentrations of acetylcholine are seen as a parabola, and 10^{-5} M may be near the inflection point of the curve.

It is generally believed that potassium and calcium induce larvae to metamorphose by depolarizing excitable cells involved in the perception of inducing stimuli or directly activating the nervous system (Yool et al. 1986; Baxter & Morse 1987; Carpizo-Ituarte & Hadfield 1998). Potassium has been proved to have effects of larval metamorphosis on *Haliotis diversicolor* Reeve, 1846 (Li et al. 2006), *Bugula neritina* (Linnaeus, 1758) (Yu et al. 2007) and *M. galloprovincialis* (Sánchez-Lazo & Martínez-Pita 2012). However, our data show that *R. venosa* larvae had no obvious inducing response to various concentrations of K^+, which is consistent with what has been reported for *M. galloprovincialis* larvae (Yang et al. 2008b), and K^+ was toxic to the larvae at high concentrations after a long exposure time.

Calcium appears to act in the signal transduction processes in both the morphogenetic and regulatory pathways (Morse 1990). Some reports about calcium showed that it had no inductive effect on *Perna viridis* (Linnaeus, 1758) (Ke et al. 1998) or *B. neritina* (Yu et al. 2007). A low concentration (5 mM) of Ca^{2+} was found to have inducing effects on larval metamorphosis of *R. venosa* in our study. However, *R. venosa* showed no induced response to excess calcium (≥5 mM), which is similar to results in a previous study on *H. rufescens* (Baloun & Morse 1984).

Conclusion

This is the first study investigating the larval metamorphosis of *Rapana venosa* in response to ionic and neuroactive compounds. In the present study, it has been shown that the larval metamorphosis of *R. venosa* can be improved by the addition of EPI,

L-DOPA, GABA, acetylcholine and $CaCl_2$. Though EPI (10^{-3} M for 6 h), L-DOPA (10^{-3} M, 10^{-4} M for 6 h) and GABA (10^{-5} M for 24 h) were proved to be effective in inducing larval metamorphosis of *R. venosa*, they also resulted in high mortalities. Hence, there is a note of caution for producers when applying EPI, L-DOPA and GABA in the artificial seeding of *R. venosa*. In contrast, acetylcholine chloride and $CaCl_2$ can induce high metamorphosis rates with low mortality rates. Thus, acetylcholine chloride and $CaCl_2$ are the most suitable among the seven chemical cues tested to be applied to the artificial seeding of *R. venosa*.

Disclosure statement

No potential conflict of interest was reported by the authors.

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