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**Induction of larval metamorphosis in the sea cucumber *Apostichopus japonicus* by neurotransmitters**

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**Abstract**

Larval metamorphosis inducers of the sea cucumber *Apostichopus japonicus* were screened from physiologically active compounds. Doliolaria larvae completed their metamorphosis to juveniles in 120 hours when treated with 5-10 μM of dopamine and L-DOPA, and 50 μM of L-adrenaline and L-noradrenaline. Doliolaria larvae had to be exposed to dopamine or L-DOPA for at least 24 h. D1-like dopamine receptor antagonists SKF87566 and LE300 (10 μM) inhibited metamorphosis by dopamine. However, the D2-like dopamine receptor antagonists sulpiride and nemonapride (10 μM) did not inhibit the effect of dopamine. The results suggested that D1-like dopamine receptors are involved in larval metamorphosis of the sea cucumber *A. japonicus*.

KEY WORDS: doliolaria larva, dopamine, L-DOPA, metamorphosis, pentactula larva

## Introduction

The demand for the sea cucumber *Apostichopus japonicus* [1] as a food product is increasing in China; and in Japan, sea cucumber cultivation has accelerated in the last decade. However, the aquaculture technique of producing sea cucumbers has not been developed completely. For example, the metamorphosis rate of sea cucumber larvae is still low. To improve the aquaculture technique, it is therefore important to understand the mechanism of larval metamorphosis.

The sea cucumber *A. japonicus* has four larval stages: gastrula (1 day after fertilization), auricularia (2-11 days), doliolaria (13-14 days), and pentactula (15 days) [2]. About 2 weeks after fertilization, the larvae change from the auricularia to the doliolaria form, then to the pentactula larvae which have five primary tentacles. Twenty days after fertilization, the pentactula larva changes to a juvenile with a tube foot and parapodium (Fig.1). In sea-cucumber hatcheries, larvae are grown to the auricularia stage by feeding them planktonic diatoms (*Chaetoceros gracilis*). Competent doliolaria larvae are then put into a tank with a substratum of periphytic diatoms, or the green alga *Ulvella lens*, which may induce metamorphosis [2].

Metamorphosis in many marine invertebrate larvae is induced by chemicals. For example, γ-aminobutyric acid (GABA) induces metamorphosis of abalone (*Haliotis rufescens*) larvae [3]. A number of chemical inducers of sea urchin larval metamorphosis have been reported; *e.g.* dibromomethane [4], eicosapentaenoic acid (EPA) [5], arachidonic acid [5], 3,3’,5,5’-tetraiodothyronine ($T_4$) [6], 3,3’,5-triiodothyronine ($T_3$) [6], glycoglycerolipids [7], L-glutamine[8], potassium chrolide

[9], dopamine [10] and L-DOPA [10]. In the tropical sea cucumber *Holothuria scabra*, leaves of the sea grass *Thalassia hemprichii* are the preferred settlement substratum. Soluble extracts of their leaves induced metamorphosis and settlement on clean plastic surfaces [11]. However, inducers of sea-cucumber larval metamorphosis have not been characterized. In this study, several compounds including neurotransmitters, amino acids and hormones have been tested for metamorphosis-inducing activity in the sea cucumber *A. japonicus*.

**Material and methods**

Larvae of the sea cucumber, *Apostichopus japonicus* were supplied by the Shikabe Branch of the Hokkaido Aquaculture Development Authority (Hokkaido, Japan). About 2 weeks after fertilization, competent doliolaria larvae were placed in 12-well tissue culture dishes (Falcon) at a density of approximately 10 larvae per well. Assays were conducted at 25 °C (dark) in 2 ml of artificial sea water (ASW) (NaCl 425 mM, KCl 9.3 mM, $CaCl_2 \cdot 2H_2O$ 10 mM, $MgCl_2 \cdot 6H_2O$ 24.5 mM, $MgSO_4 \cdot 7H_2O$ 25.5 mM, $NaHCO_3$ 2.5 mM). In all experiments, larvae were periodically examined with a dissecting microscope to monitor morphology patterns in response to the test chemicals. As a negative control, larvae were observed in ASW without any test chemical. A film of the green alga *Ulvella lens* (0.5 $cm^2$/ml) was used as a positive control. The algal film treated with antibiotics (streptomycin 30 μg/ml and penicillin G 20 μg/ml) was also tested.

The chemicals tested were; dopamine hydrochloride and L-noradrenaline (Wako Pure Chemical

Industries, Ltd.), L-3,4-dihydroxyphenylalanine (L-DOPA), L-adrenaline (Kanto Chemical Co., Inc.), acetylcholine chloride, γ-aminobutyric acid and reserpine (Wako Pure Chemical Industries, Ltd.), serotonin (Nacalai Tesque, Inc.), γ-aminovaleric acid (Aldrich), octopamine and tyramine (Sigma), 22 proteinaceous amino acids (Sigma), taurine (Sigma), L-thyroxine (Nacalai Tesque, Inc.), D-thyroxine (ICN), L-3,3',5-triiodothyronine (Tokyo Chemical Industry Co., Ltd.), 20-hydroxyecdysone (ICN Biochemicals), dibromomethane, phenethylamine (Kanto Chemical Co., Inc.), potassium chloride, EPA and diphenhydramine hydrochloride (Nacalai Tesque, Inc.), atropine and histamine (Wako Pure Chemical Industries, Ltd.). All were dissolved in distilled water and diluted more than 10-fold with ASW to give final concentrations of 10 mM, 1 mM, 100 μM. Furthermore the active chemicals were tested at various concentrations.

Three types of experiments were conducted. The first set of experiments was designed to determine the ability of selected chemicals to induce metamorphosis. The larvae were exposed throughout the experiment for 120 h to the various concentrations of the test compounds. The second set of experiments was designed to determine the minimum time required for the larvae to be irreversibly affected by a test solution. Larvae were exposed to a test compound for different periods of time (exposure time). The larvae were then rinsed twice with ASW, followed by replacement in fresh ASW. The third set of experiments was designed to assess the effects of dopamine receptor antagonists. Dopamine D1-like receptor antagonists SKF83556 hydrobromide and LE300 (Tocris Cookson, Ltd.), and dopamine D2-like receptor antagonists sulpiride (Wako Pure Chemical Industries, Ltd.) and nemonapride (Tocris Cookson, Ltd.) were dissolved in ASW. After the larvae were exposed to each

dopamine antagonist (10 and 1 μM) and dopamine (10 μM) for 120 h, the rate of metamorphosis was estimated.

In the first experiment, all wells were examined to determine the percentages of the growth stages after 12, 24, 36, 48, 60, 72, 96 and 120 h. A sea-cucumber larva was categorized as an early pentactula larva if tentacles were present, as a late pentactula larva if a tube foot was present, and as a juvenile if it possessed a tube foot and parapodium. In the second experiments, all wells were examined to determine the percentage of juveniles. The result was determined after 96 h. All treatments in each experiment were replicated at least in triplicate. In the third experiments, all wells were examined to determine the percentages of the growth stages after 24, 48, 72, 96 and 120 h. Data for larval metamorphosis of sea cucumbers were analyzed by one-way ANOVA followed by Tukey's multiple range test between ASW and inducers. Differences were considered significant at $P < 0.01$.

**Results**

Most of untreated doliolaria larvae did not undergo metamorphosis to the pentactula stage. Nevertheless, some of the larvae had changed to the early pentactula (36 %) and late pentactula (4 %) stages after 120 h. Only a small amount of larvae (6 %) metamorphosed to juveniles (Fig. 2a). *Ulvella* film induced larval metamorphosis of *Apostichopus japonicus*. Pentactula larvae began to appear after 12 h and to settle after 24 h by algal induction. Juveniles with parapodium were observed after 60 h. After 120 h, 87 % of larvae changed to the juvenile form (Fig. 2b). *Ulvella* film treated with antibiotics

also induced metamorphosis of 80 % of the larvae (data are not shown).

Forty-five compounds including amino acids, and well-known metamorphosis inducers of marine invertebrates like GABA, were tested for metamorphosis-inducing activity of *A. japonicus*. No amino acid showed either metamorphosis induction or toxicity even at 10 mM. Neuroactive compounds such as histamine and serotonin, as well as metamorphosis related thyroid hormones, were inactive and non-toxic. Only dopamine, L-DOPA, L-adrenaline and L-noradrenaline induced metamorphosis of the sea cucumber larvae at 100 μM, although they caused toxic effects at 10 mM and 1 mM.

Dopamine, L-DOPA, L-adrenaline and L-noradrenaline were tested further at 10 μM (Fig. 2c-f). Most of the larvae changed to the pentactula stage after 24 h when treated with dopamine (90 %) and L-DOPA (88 %). Dopamine and L-DOPA induced metamorphosis to the pentactula stage slightly earlier than the *Ulvella* film. However, larvae changed to the juvenile form when subjected to dopamine and L-DOPA at a similar time as the *Ulvella* film. Finally, almost all larvae changed to juveniles in dopamine (96 %) and L-DOPA (98 %), although a few percent of larvae died after 120 h. In L-adrenaline and L-noradrenaline, only half of the larvae metamorphosed to the pentactula stage after 24 h. The rest of the larvae remained at the doliolaria stage, even after 120 h in the case of L-noradrenaline. The metamorphosis rate to juveniles after 120 h was 23 % in L-adrenaline and 25 % in L-noradrenaline. The morphology in each of the larval stages was similar between *Ulvella* film, dopamine and other neurotransmitters (Fig. 3).

These neurotransmitters were tested at various concentrations to reveal the minimum effective concentrations (Fig. 4). Dopamine induced larval metamorphosis (83 %) at a concentration of 5 μM

after 120 h. It did not induce metamorphosis at less than 2.5 μM. L-DOPA was slightly less active (60 %) at 5 μM, and was inactive at 2.5 μM. L-adrenaline and L-noradrenaline showed potent metamorphosis induction at 50 μM. However, they showed only weak activity at 20 μM. Each treatment with neurotransmitters was analyzed by one-way ANOVA with ASW. The percentage of juveniles after 120 hours was significantly different in dopamine and L-DOPA at a concentration of more than 5 μM, and L-adrenaline and L-noradrenaline at a concentration of more than 20 μM (Tukey's test at $P < 0.01$) (Fig. 4).

Minimum exposure time of the test compounds was established for dopamine and L-DOPA at 10 μM (Fig. 5). An exposure time less than 12 hours to both compounds was not enough to induce metamorphosis of sea-cucumber larvae. To metamorphose, larvae needed at least 24 h exposure to both compounds. Most of the larvae changed to pentactula larvae in 24 h when treated with dopamine or L-DOPA. Therefore doliolaria larvae have to be exposed to dopamine and L-DOPA until metamorphosis to the pentactula stage. After larvae were changed to the pentactula stage, they do not have to be exposed, or have to be exposed for only a short period, to inducers.

The effect of dopamine receptor antagonists is summarized in Fig. 6. Dopamine D1-like receptor antagonists (SKF83566 and LE300) and D2-like receptor antagonists (sulpiride and nemonapride) were assessed. Both SKF83556 and LE300 at 10 μM antagonized the effect of dopamine (10 μM). They were, however, inactive at 1 μM. Sulpiride and nemonapride did not inhibit metamorphosis in the presence of dopamine.

**Discussion**

In our experiment, untreated larvae did not complete metamorphosis, while *Ulvella* film was confirmed to induce metamorphosis. The effect of *Ulvella* was not related to microbial organisms.

Dopamine, L-DOPA, L-adrenaline and L-noradrenaline induced larval metamorphosis in *Apostichopus japonicus*. Dopamine and L-DOPA were especially active at $5\times10^{-6}$ M. In the sea urchin *Dendraster excentricus*, metamorphosis was induced by dopamine at a concentration of $10^{-5}$ M [10]. However, dopamine only affected a small proportion of larvae. Metamorphosis and settlement of other marine invertebrate larvae is also induced by these neurotransmitters (Table 1). Dopamine ($10^{-4}$ M), L-DOPA ($10^{-4}$ M) and noradrenaline ($10^{-5}$ M) were reported as inducers of larval metamorphosis in the hydrozoan *Halocordyle disticha*, while adrenaline was inactive [12]. The catecholamine precursors phenylalanine and tyrosine were not found to induce metamorphosis in *H. disticha*. In addition none of these compounds was effective in the hydrozoan *Hydractinia echinata*. In the polycheate *Hydroides ezoensis*, L-DOPA ($3\times10^{-6}$ M), L-adrenaline ($3\times10^{-5}$ M) and L-noradrenaline ($1\times10^{-4}$ M) showed inductive activity of larval metamorphosis and were toxic at higher concentrations [13]. Dopamine only showed toxicity at $1\times10^{-5}$- $3\times10^{-4}$ M in *H. ezoensis*. In the polycheate *Pomatoleios kraussii*, the same three neurotransmitters were metamorphosis inducers, although only L-DOPA induced metamorphosis in the polycheate *Ficopomatus enigmaticus* at $3\times10^{-6}$ M [13]. The reaction was delayed and tube formation was absent in the case of metamorphosis by L-DOPA in *H. ezoensis*. Chevolot et al. reported L-DOPA ($5.1\times10^{-3}$ M) and L-adrenaline ($5.5\times10^{-3}$ M) induced metamorphosis of the larvae of the

scallop *Pecten maximus* (5-15 %) [14]. They suggested that quinones transformed from these catecholamines showed activity and an alga-derived quinone, or quinone-like structure, is the true inducer present in the environment. In the nudibranch *Phestilla sibogae*, dopamine (22 % at $10^{-5}$ M) and L-adrenaline (12 % at $10^{-5}$ M) elicited partial metamorphosis [15]. In the oyster *Crassostrea gigas*, L-DOPA ($10^{-5}$ M) induced both settlement and metamorphosis [16]. L-adrenaline ($10^{-4}$ M) and L-noradrenaline ($10^{-4}$ M) induced metamorphosis only, without settlement. In the barnacle *Balanus amphitrite*, dopamine ($1.3\times10^{-4}$ M) and L-DOPA ($3.2\times10^{-5}$ M) induced settlement, but did not induce metamorphosis of larvae [17]. On the other hand, settlement of the bryozoan *Bugula neritina* was inhibited by dopamine at a concentration of $10^{-4}$ M [18].

As pointed out above, four neurotransmitters induced abnormal larval metamorphosis in some invertebrates. In contrast, sea-cucumber metamorphosis by these neurotransmitters was similar to *Ulvella* film treatment. Sea-cucumber larval metamorphosis was induced by a set of four compounds only, dopamine, L-DOPA, L-adrenaline and L-noradrenaline. However, these neurotransmitters are unlikely excreted from *Ulvella* film. Further experiments are needed to know that neurotransmitter agonists or the other chemical cue are excreted from *Ulvella lens*.

Dopamine induced sea cucumber larval metamorphosis at a low concentration (5 μM), and metamorphosis rate was high (83 %). However, dopamine and L-DOPA needed 24-h exposure and a 96-h period to induce complete metamorphosis. Their effects on *A. japonicus* are slower than most other invertebrate examples. In 24 h, these inducers changed larvae to the late pentactula stage. It is suggested that these inducers activate the change from the doliolaria stage to tube-foot formation (late pentactula

larvae). The pentactula larvae could metamorphose to the juvenile stage without a chemical cue. The adult nervous system develops during the doliolaria stage [19]. The neurotransmitter may interact with the nervous system in the late doliolaria. Dopamine and L-DOPA might need to be exposed for a short time at a certain stage of doliolaria development.

Dopamine D1-like receptor antagonists SKF83566 and LE300 inhibited sea cucumber metamorphosis by dopamine, while D2-like receptor antagonists did not. In mammals, there are two types of D1-like receptors, the D1 receptor and D5 receptor. Both receptors enhanced cAMP level in nerve cells [20]. In the barnacle *Balanus amphitrite*, two signal transduction systems, the adenylate cyclase/cyclic AMP (AC/cAMP) pathway [21, 22] and the PI/DAG/PKC pathway [23], appear to regulate metamorphosis. The enhancement of cAMP was suggested to be a signal in sea-cucumber metamorphosis.

In conclusion, our results indicated that dopamine and L-DOPA are endogenous larval metamorphosis inducers of the sea cucumber *A. japonicus*. Dopamine interacted with dopamine D1-like receptors.

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cucumber.

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**Figure captions**

**Fig. 1** The growth stages of the sea cucumber, *Apostichopus japonicus*. Scale bars 100 μm. (a) Auricularia larva, (b) Doliolaria larva, (c) Early pentactula larva, (d) Late pentactula larva, (e) Juvenile (*Ten*: tentacle, *Tub*: Tube foot, *P*: Parapodium)

**Fig. 2** Percentages of each larval stage of *Apostichopus japonicus*, (a) Artificial sea water (ASW) (n=7), (b) Green alga *Ulvella lens* film (0.5 $cm^2$/ml) (n=7), (c) Dopamine 10 μM (n=7), (d) L-DOPA 10 μM (n=7), (e) L-adrenaline 10 μM (n=7), (f) L-noradrenaline 10 μM (n=7)

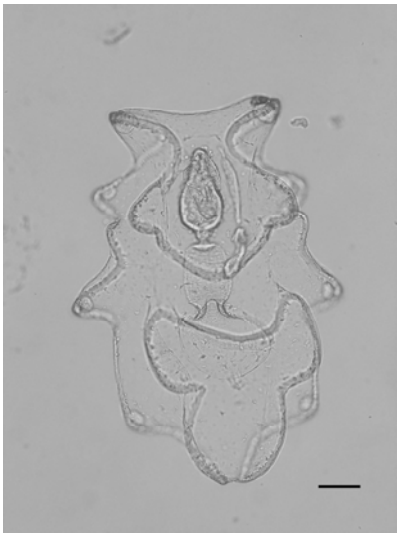
**Fig. 3** Morphological changes of *Apostichopus japonicus* larvae when treated with dopamine (5 μM), after exposure for (a) 3h , (b) 24h, (c) 40h, (d) 60h, (e) 120h (*Ten*: tentacle, *Tub*: Tube foot). Scale bars 100 μm

**Fig. 4** Mean percentage of juvenile *Apostichopus japonicus* after 120 h. *Error bars* indicates standard error. ** $P < 0.01$

**Fig. 5** The effect of exposure time, (a) Dopamine, 10 μM (n=3) and (b) L-DOPA, 10 μM (n=3). *Error bars* indicate standard error

**Fig. 6** Effect of dopamine receptor antagonists after 120 h. (a) antagonists at 10 μM. (b) antagonists at 1 μM with dopamine (DA) (10 μM). *Error bars* indicate standard error. *Different letters* indicate significantly differences at $P < 0.01$

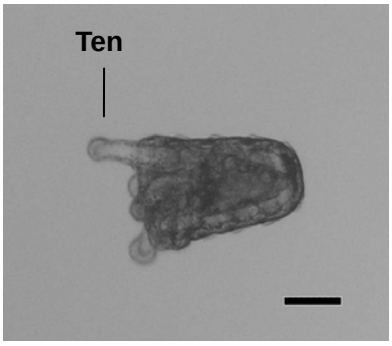
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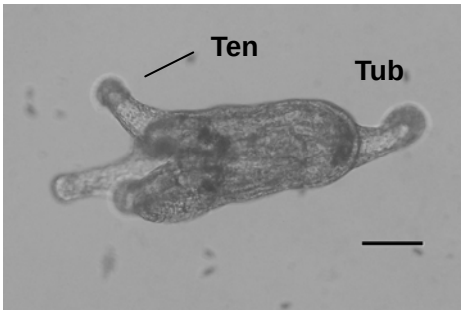
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(c)




(d)




(e)

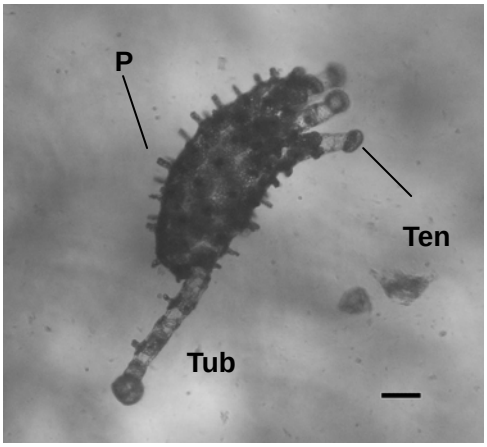
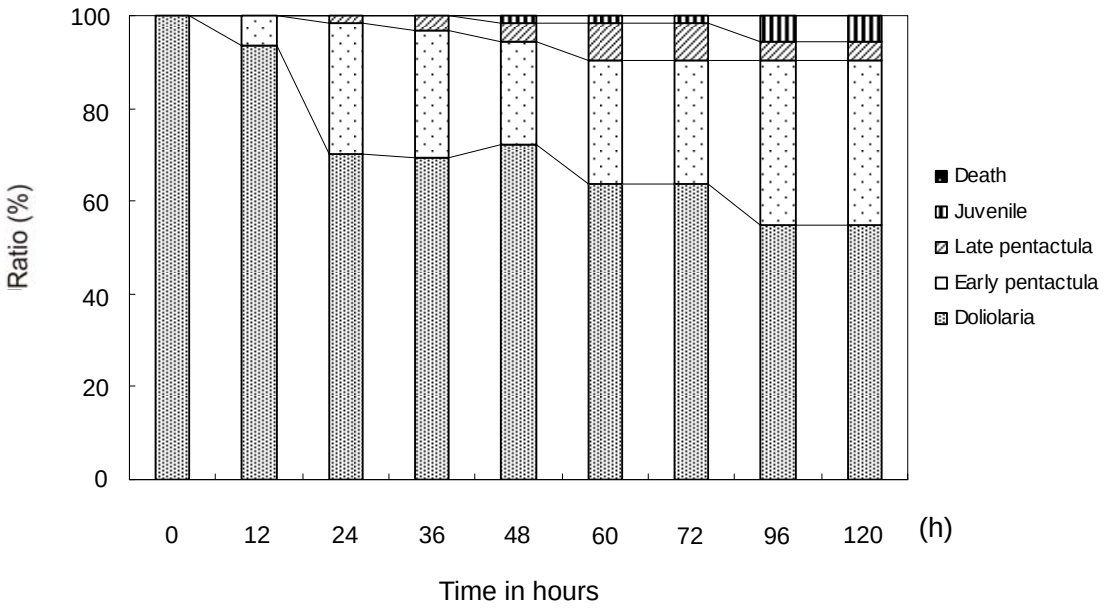
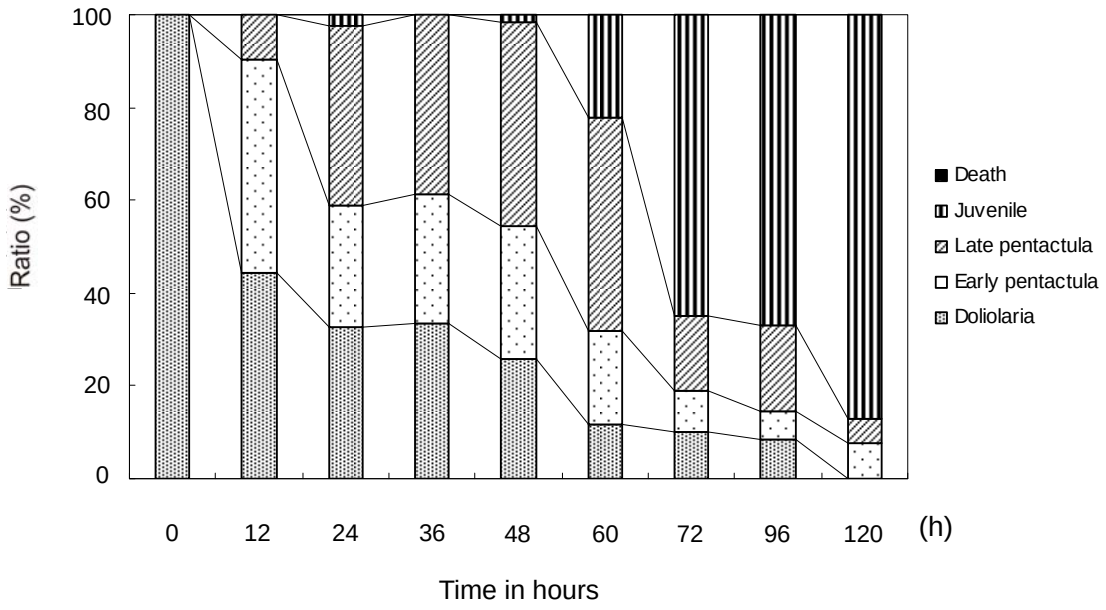



Fig. 1

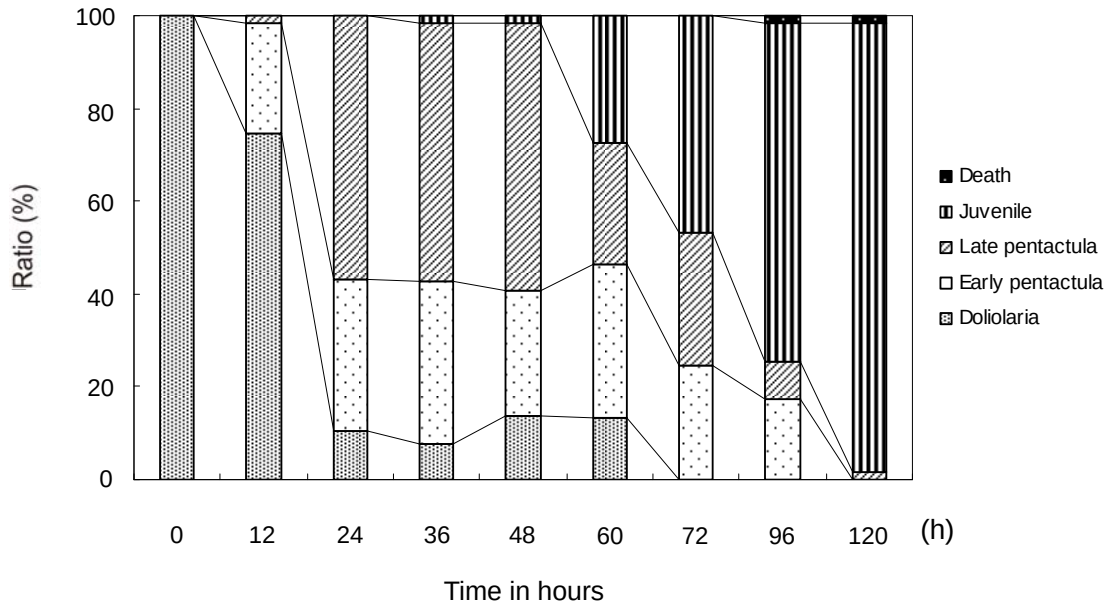
(a)


100
80
60
40
20
0
Ratio (%)
0
12
24
36
48
60
72
96
120
(h)
Time in hours
Death
Juvenile
Late pentactula
Early pentactula
Doliolaria

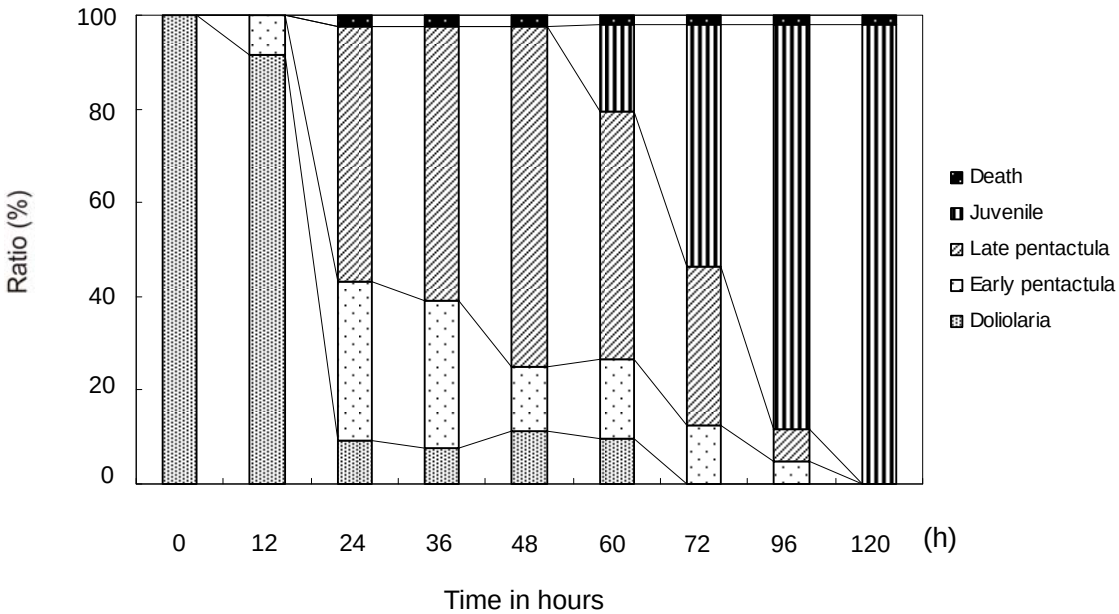
(b)


100
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Ratio (%)
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12
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72
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(h)
Time in hours
Death
Juvenile
Late pentactula
Early pentactula
Doliolaria

(c)


100
80
60
40
20
0
Ratio (%)
0
12
24
36
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60
72
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(h)
Time in hours
Death
Juvenile
Late pentactula
Early pentactula
Doliolaria

(d)


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Ratio (%)
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Time in hours
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Juvenile
Late pentactula
Early pentactula
Doliolaria

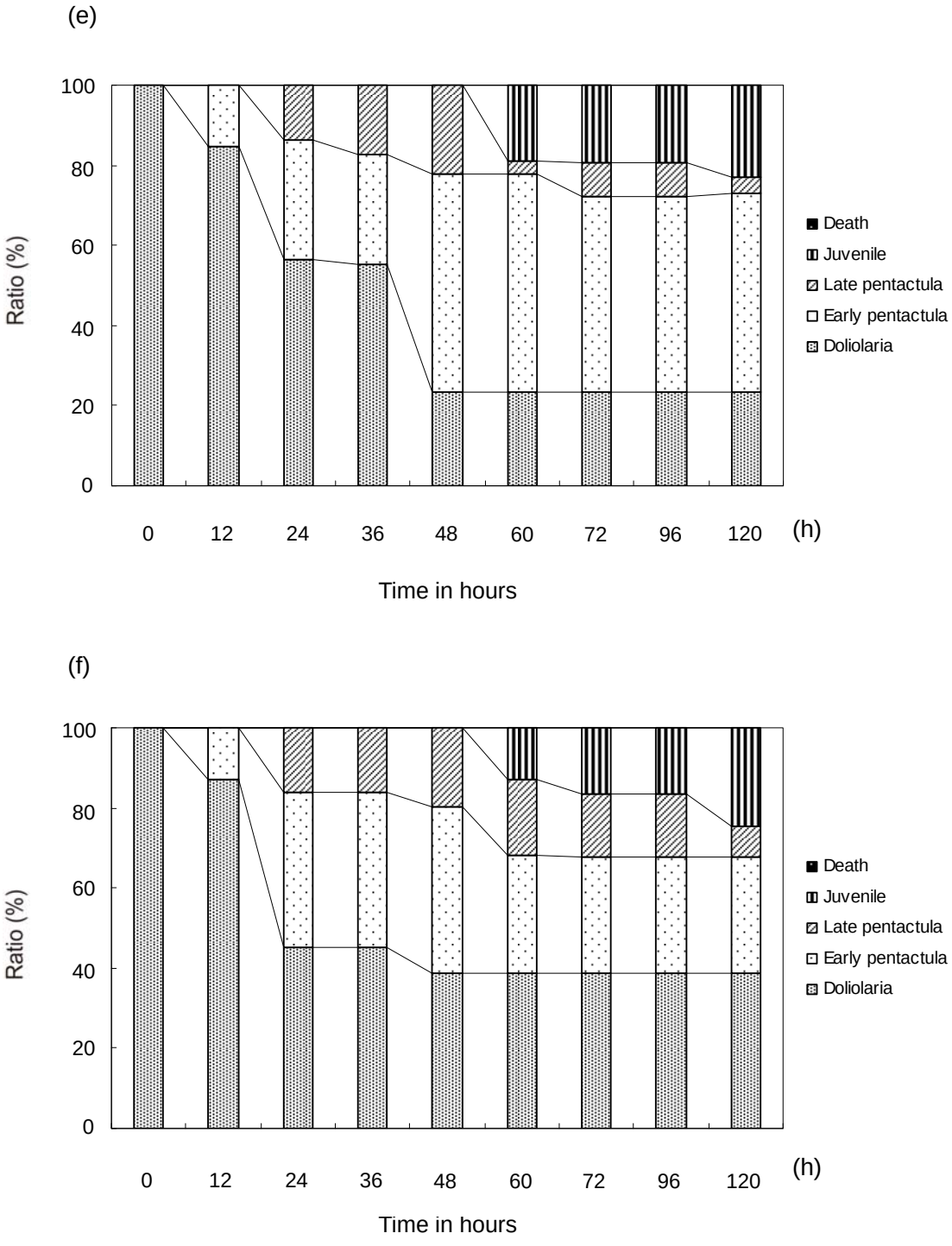

(e)
100
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Ratio (%)
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Time in hours
Death
Juvenile
Late pentactula
Early pentactula
Doliolaria
(f)
100
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Ratio (%)
0
12
24
36
48
60
72
96
120
(h)
Time in hours
Death
Juvenile
Late pentactula
Early pentactula
Doliolaria

Fig. 2

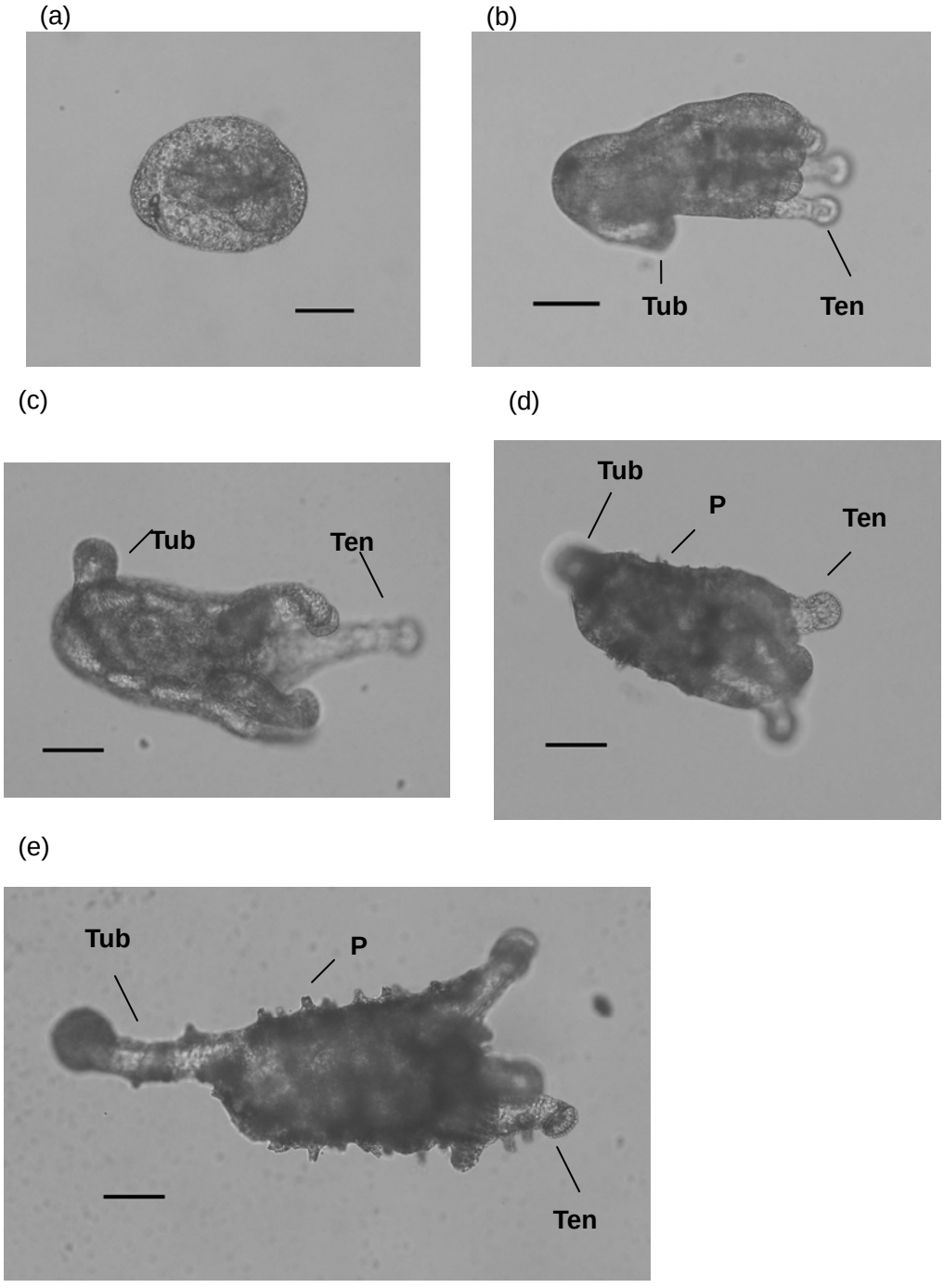



Fig. 3

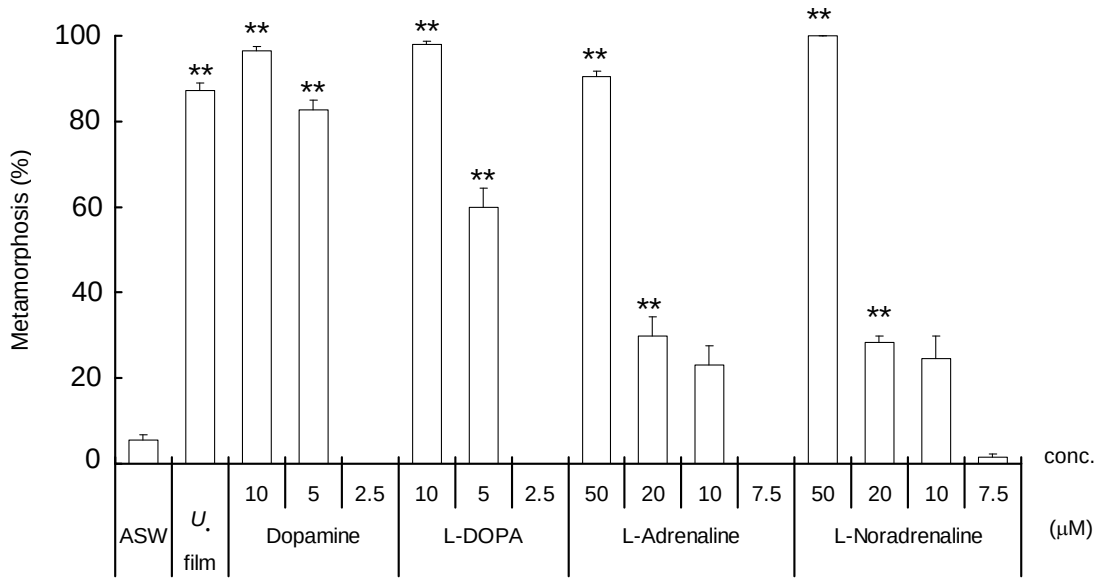
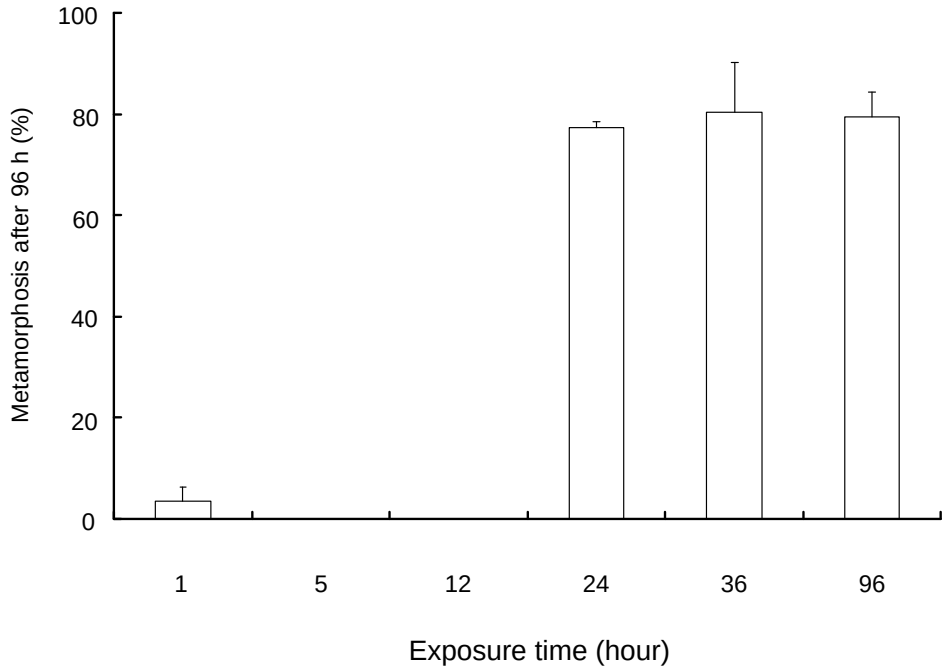

Metamorphosis (%)
100
80
60
40
20
0
**
conc.
10
5
2.5
50
20
7.5
(μM)
ASW
U. film
Dopamine
L-DOPA
L-Adrenaline
L-Noradrenaline

Fig. 4

(a)


100
80
60
40
20
0
Metamorphosis after 96 h (%)
1
5
12
24
36
96
Exposure time (hour)

(b)

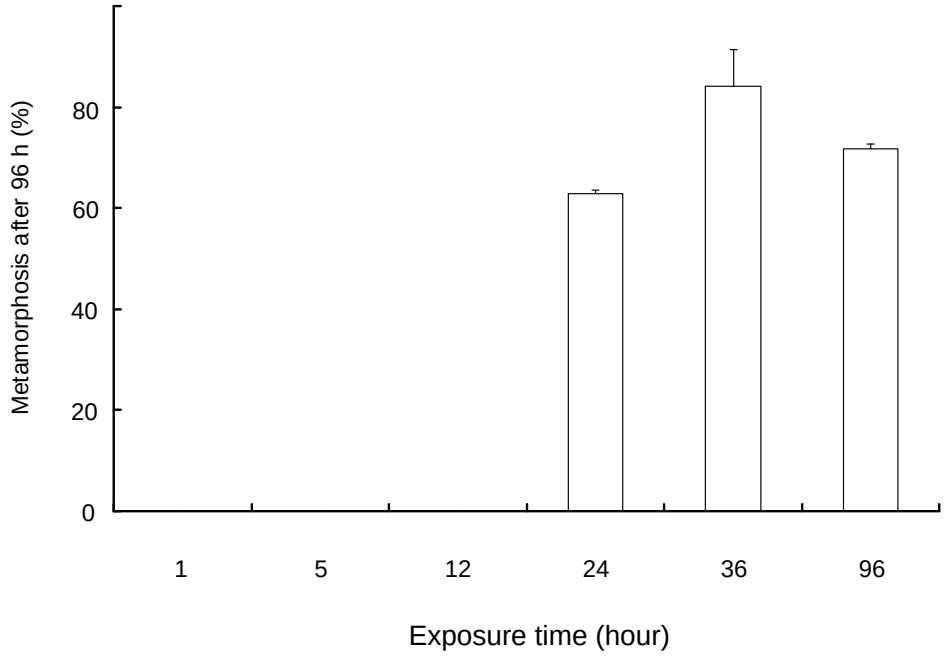
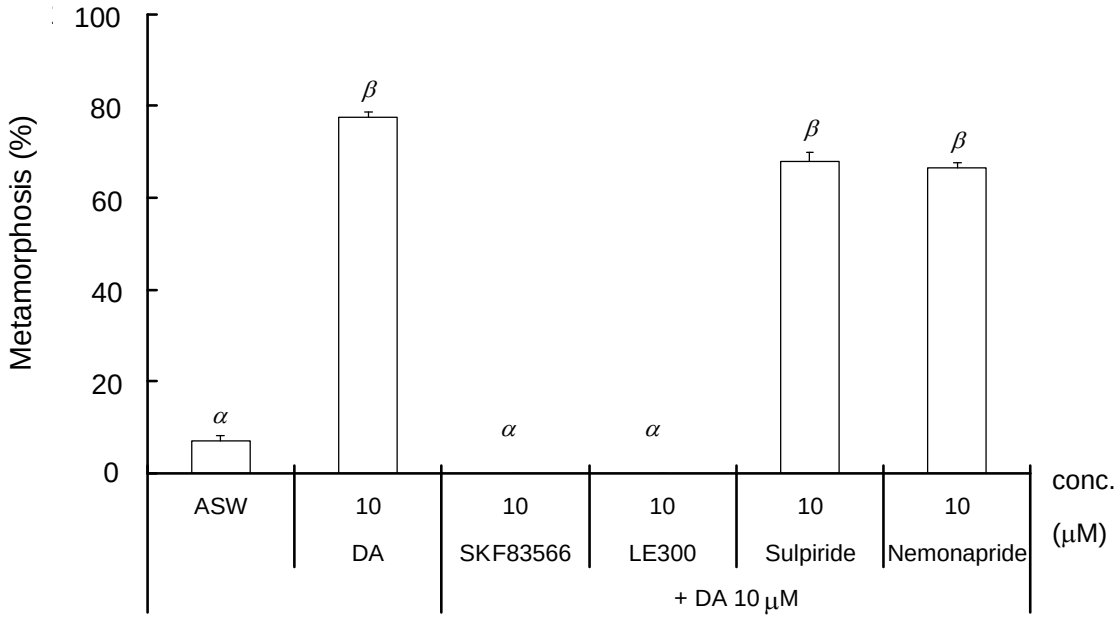

80
60
40
20
0
Metamorphosis after 96 h (%)
1
5
12
24
36
96
Exposure time (hour)

Fig. 5

(a)


Metamorphosis (%)
100
80
60
40
20
0
β
α
ASW
10
DA
10
SKF83566
10
LE300
10
Sulpiride
10
Nemonapride
conc.
(μM)
+ DA 10 μM

(b)

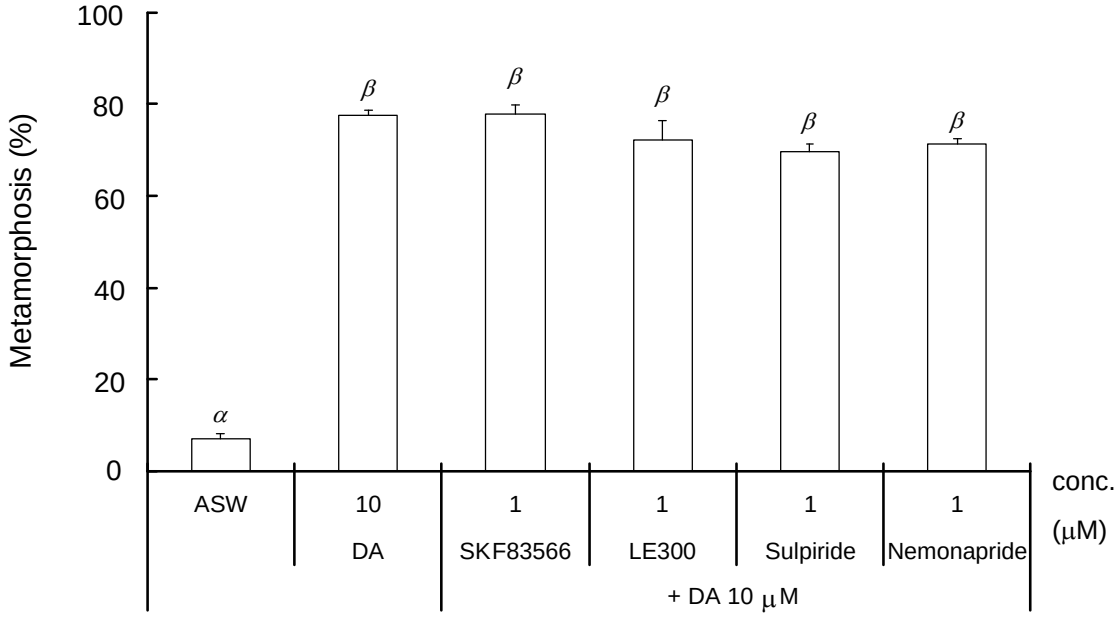

Metamorphosis (%)
100
80
60
40
20
0
β
α
ASW
10
DA
1
SKF83566
1
LE300
1
Sulpiride
1
Nemonapride
conc.
(μM)
+ DA 10 μM

Fig. 6

Table 1 Metamorphosis-inducing minimum concentrations of neurotransmitters in marine invertebrate larvae

| Species | Dopamine | L-DOPA | Adrenaline | Noradrenaline | References |
|---|---|---|---|---|---|
| *Halocordyle disticha* (Cn) | $10^{-4}$ M (93 %) | $10^{-4}$ M (97 %) | no effect | $10^{-5}$ M (70 %) | [12] |
| *Hydractinia echinata* (Cn) | no effect | no effect | no effect | no effect | [12] |
| *Pecten maximus* (Mo) | - | $5.1\times10^{-3}$ M (5 %) | $5.5\times10^{-3}$ M (12 %) | no effect | [14] |
| *Phestilla sibogae* (Mo) | $10^{-5}$ M[a] (22 % ) | - | $10^{-5}$ M[a] (12 %) | no effect | [15] |
| *Crassostrea gigas* (Mo) | no effect | $10^{-5}$ M | $10^{-4}$ M[b] | $10^{-4}$ M[b] | [16] |
| *Bugula neritina* (Br) | $10^{-4}$ M (inhibition) | - | - | - | [18] |
| *Hydroides ezoensis* (An) | no effect | $3\times10^{-6}$ M | $3\times10^{-5}$ M | $10^{-4}$ M | [13] |
| *Pomatoleios kraussii* (An) | no effect | $3\times10^{-5}$ M | $10^{-4}$ M | $10^{-4}$ M | [13] |
| *Ficopomatus enigmaticus* (An) | no effect | $3\times10^{-6}$ M | - | - | [13] |
| *Balanus amphitrite* (Ar) | $1.3\times10^{-4}$ M0[c] | $3.2\times10^{-5}$ M[c] | - | - | [17] |
| *Dendraster excentricus* (Ec) | $10^{-5}$ M (25%) | no effect | - | no effect | [10] |
| ***Apostichopus japonicus* (Ec)** | **$5\times10^{-6}$ M (83 %)** | **$5\times10^{-6}$ M (60 %)** | **$10^{-5}$ M (23 %)** | **$10^{-5}$ M (25 %)** | **Present study** |

*Cn* Cnidaria, *Mo* Mollusca, *Br* Bryozoa, *An* Annelida, *Ar* Arthropoda, *Ec* Echinodermate, - not tested

[a] Induced partial metamorphosis

[b] Induced only metamorphosis without settlement

[c] Induced only settlement without metamorphosis