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Effects of pharmacological compounds on the barnacle larval development, metabolism and settlement

Ali M. Al-Aidaroos, S. Satheesh*, Reny P. Devassy

Department of Marine Biology, Faculty of Marine Sciences, King Abdulaziz University, Jeddah, Saudi Arabia

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ABSTRACT

Effects of select pharmacological compounds, with known mode of action in vertebrates, were evaluated on the development, metabolism and settlement of larvae of the common fouling barnacle, *Amphibalanus amphitrite*. Atrovastatin, a lipid-regulating compound, cetrizine hydrochloride, an anti-histamine, atenolol, a β-blocker, and amlodipine, a calcium-channel blocker were the compounds studied. Nauplii treated with these compounds took more days to reach the cypris stage when compared with the control. These compounds also inhibited the settlement of cyprids on Petri dishes. While exposure to these compounds led to a decrease in the metabolic activity of stage III nauplii, it increased the respiratory rate of cyprids. The results emphasize the role of neurotransmitters and lipids in the development and settlement of barnacle larvae on hard surfaces.

1. Introduction

Biofouling is a common process in the marine environment by which natural or artificial substrata are colonized by both microorganisms and macroorganisms (Clare, 1996; Dobretsov et al., 2015). Barnacles are the most important macrofouling organisms settling on objects submerged in marine waters (Satheesh and Wesley, 2008, 2009; Maréchal and Hellio, 2011). The life cycle of barnacle includes six naupliar stages and a settling cyprid stage (Zhang et al., 2010; Maréchal et al., 2012). Cyprids do not feed and mainly depend on storage proteins and lipids for their physiological activity. The cypris larva of the barnacle also have a well-developed nervous system with a small brain for coordination (Harrison and Sandeman, 1999). This nervous system helps the cypris larvae for select a suitable substratum for settlement (Dahms et al., 2004). Studying the factors that influence the life cycle of barnacles is important for understanding the developmental biology and community ecology of these organisms and for formulating effective antifouling measures (Vogan et al., 2003; Dahms et al., 2004).

Settlement on hard substratum by invertebrate larvae depends on many factors (Berntsson et al., 2000; Chan et al., 2014). Most larvae are capable of sensing the environment/substrata and responding to factors such as surface roughness, biofilms of conspecific adults, etc. (Maki et al., 1990; Berntsson et al., 2000; Zega et al., 2007; Li et al., 2014; Yang et al., 2014). The mechanism of larval settlement and later metamorphosis on substrata are the subjects of interest of marine ecologists and marine technologists. This is especially due to the fact that the mechanism of larval settlement remains unclear for most fouling organisms (Jin et al., 2014). Pharmacological compounds have been used as tools to find out signal pathways in larval settlement (Clare and Matsumura, 2000; Dahms et al., 2004; Tanur et al., 2010; Jin et al., 2014). These compounds induce or inhibit the settlement of many marine invertebrates (Yamamoto et al., 1996; Dahms et al., 2004; Gohad et al., 2010; Chai et al., 2014).

Neurotransmitters such as serotonin, epinephrine, dopamine, acetylcholine and histamine influence barnacle larval settlement (Yamamoto et al., 1996; Dahlstrom et al., 2005; Holm, 2012; Jin et al., 2014). Pharmacological compounds which interrupt signaling processes by acting against these neurotransmitters may inhibit larval settlement on surfaces (Dahms et al., 2004). For example, noradrenaline and medetomidine (both having antagonistic activity against epinephrine) exhibit strong antifouling activity against barnacles (Jin et al., 2014). Knowledge of the mode of

* Corresponding author.
E-mail addresses: ssathianeson@kau.edu.sa, satheesh_s2005@yahoo.co.in (S. Satheesh).

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action of these compounds on the target organism is important for possible application as antifouling agents.

In this study, conducted under laboratory conditions, select pharmacological compounds were evaluated on the development, metabolism and settlement of the larval stages of *Amphibalanus amphitrite* (=*Balanus amphitrite*), a dominant barnacle species among the fouling communities found inhabiting hard surfaces in tropical and subtropical waters (Qiu et al., 2005; Satheesh and Wesley, 2009). Larval forms of *A. amphitrite* are widely used as model organisms for antifouling screening and ecotoxicological studies due to their sensitivity to toxicants, short larval development duration and easy availability (Wu et al., 1997; Anil et al., 2010). Although barnacle larval settlement and metamorphosis have been studied in detail, not many studies have been carried out on the effects of pharmaceutical compounds, which are potential antifoulants. Results obtained in this study will improve our knowledge on the internal biochemical pathways, particularly ligand-receptor interactions and signal transduction systems which regulate larval settlement and metamorphosis.

2. Materials and methods

2.1. Collection and maintenance of adult barnacles

Amphibalanus amphitrite adults were collected from the Obhur Creek (near to King Abdulaziz University Marine Station at Obhur) on the Jeddah coast of the Red Sea and brought to the laboratory in a bucket with seawater. In the laboratory, adult barnacles were maintained in filtered (Millipore, 47 μm), aerated seawater. The barnacles were fed a mixed algal diet, which included *Tetraselmis* and *Thalassiosira* (2.5×10^5 cells ml^{-1}). Adult barnacles normally released nauplii within 1–3 days of collection. Stage II nauplii were collected using a net (mesh size 100 μm) with the help of a light source. Collected nauplii were transferred to small troughs with filtered seawater using Pasteur pipettes.

2.2. Pharmacological compounds

Atrovastatin (10 mg), a lipid-regulator, cetrizine hydrochloride (10 mg), an antihistamine, atenolol (50 mg), a β-blocker and amlodipine (5 mg), a calcium-channel blocker were selected for this study. Table 1 shows the list of pharmaceutical compounds used in this experiment along with their mode of action. These compounds were dissolved in filtered seawater for the preparation of stock solution.

2.3. Larval rearing for toxicity and settlement assays

Nauplii were kept in small troughs with mild aeration in a controlled environment chamber (walk-in type) at 27 °C. They were fed microalgal cultures, *Tetraselmis* and *Thalassiosira* (2.5×10^5 cells ml^{-1}). For toxicity and respiration assays, the nauplii were reared up to stage III and for settlement assays they were reared up to the cypris stage.

2.4. Experiment 1: toxicity of pharmacological compounds on nauplii

The 96-h LC_{50} value for each pharmaceutical compound was determined by performing toxicity assays (in replicate, n = 3) in six-well plates (Falcon). Stage III nauplii (10 individuals) were introduced into the wells filled with filtered seawater and kept in an environmental chamber under 12:12 h light:dark cycle. The compounds were added to the wells in different concentrations (by diluting the stock solution in filtered seawater). Dead nauplii in each well were counted every 3 h and the experiment was conducted for 96 h. Controls were maintained without the addition of any compounds. The LC_{50} values for the compounds were calculated from the mortality counts by using the regression slope. The sublethal concentration for each compound was calculated by considering 1/5th of the LC_{50} value.

2.5. Experiment 2: effects of pharmaceutical compounds on larval development duration

This experiment was designed (in replicate, n = 3) to evaluate the effect of pharmaceuticals and ions on the duration of larval development from stage II to cypris. Stage II nauplii (about 100) were transferred to 2-L jars with filtered seawater. Pharmaceuticals were added to the jars at sublethal concentrations (1/5th of LC_{50}). Controls were maintained without the addition of compounds. Nauplii were fed *Thalassiosira* (approximately 2.5×10^5 cells ml^{-1}) and maintained at 16:8 h light:dark cycle. Larval development was followed every day by checking the nauplii under dissecting microscope (Leica M80). Naupliar stages were recognized based on the general descriptions of larval forms of balanomorph barnacles (Arnsberg, 2001). The exposure medium in the jar was renewed every day up to the appearance of cyprids. Larval development duration was counted from the commencement of the experiment to the day on which 50% cyprids were observed.

2.6. Experiment 3: respiration measurement

The oxygen consumption rate of pharmaceutical compound-treated and -untreated larvae (both nauplii and cyprids) was measured by using oxygen sensors (Oxy Mini, Presense). About 20 nauplii or cyprids (in replicate, n = 3 for nauplii, n = 2 for cyprids) were transferred to Petri dishes and sublethal concentrations of the pharmaceutical compounds added. Petri dishes without pharmaceuticals were considered as control. The Petri dishes were maintained for 1 h at 27 °C under dark condition. After that, the larvae were transferred to small glass vials (known volume) with filtered seawater. The dissolved oxygen content of the medium was measured at the beginning and after 1 h at 25 °C. The rate of oxygen consumption was calculated for each larva and expressed as μg O_2 $individual^{-1}$ h^{-1}.

2.7. Experiment 4: effects of pharmaceutical compounds on settlement of cyprids

Settlement assay was carried out in polystyrene Petri dishes

Table 1
Pharmacological compounds and their mode of action in vertebrates.

Sl. No	Pharmacological compounds	Brand name	Mode of action
1	Cetirizine hydrochloride	Artiz	Antihistamine, reduces the level of natural chemical called histamine. It is a selective H_1 receptor antagonist.
2	Atrovastatin	Atorva	It is a member of medicine known as statins used for regualting lipids. Competitive inhibitor of HMG-CoA reductase.
3	Atenolol	Tenormin	Selective β1 receptor antagonist, belongs to beta blockers group.
4	Amlodipine	Amlor	It is a long acting dihydropyridine calcium channel blocker.