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# Ascidian Larvae Discriminate Nano-scale Difference in Surface Structures During Substrate Selection for Settlement

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**Planktonic larvae of sessile metazoans select substrates for settlement based on various factors. *Phallusia philippinensis* larvae (Ascidiacea: Phlebobranchia: Ascidiidae) showed a negative preference for nano-scale nipple arrays (dense arrays of papillae-like nanostructures approximately 100 nm in height). To clarify whether ascidian larvae discriminate between nano-structure sizes for substrate selection, three different sizes of periodic nano-folds were fabricated using two-beam interference exposure, and substrate selection assays were performed on the three types of nano-folds and flat surfaces made of the same material. The substrate selection assay with 500–2000 freshly hatched larvae was carried out in nine replicates. The ascidian larvae showed a positive preference for flat surfaces and a negative preference for substrates with a height of 120 nm and pitch of 600 nm. Manly's selection indices differed with the size of the periodic nano-folds, supporting the hypothesis that larvae directly or indirectly discriminate between nano-scale differences upon settlement. The present study is the first to show that differences in nanostructure size affect substrate selection during larval settlement of sessile animals. The evolutionary adaptive reasons for larvae to discriminate between nano-scale structures and select substrates for settlement are potentially important to effectively manage ascidian biofouling using non-toxic methods.**

**Key words:** ascidian larva, biofouling, nano-scale fabrication, periodic nano-folds, substrate preference, two-beam interference exposure

## INTRODUCTION

Dispersal is an important event for the survival of any organism against environmental changes (e.g., Travis et al., 2013); thus, even sessile metazoans usually have dispersal forms (e.g., larvae) in their life cycle. The larvae swim or drift in the water column, eventually attach to suitable substrates, and metamorphose into sessile forms. Substrate selection for sessile organisms is usually deterministic, because they are unable to leave the substrate and choose a different substrate following metamorphosis. Moreover, early post-settlement mortality is influenced by several factors that are typically linked to the microenvironment, such as disturbances, physiological stress, competition, and predation (Hunt and Scheibling, 1997). Therefore, the choice of the substrate is crucial for these organisms. For example, ascidian larvae generally exhibit negative phototaxis during the later part of larval life and settle on shaded sites (Tsuda et al., 2003; Salas et al., 2018), which is likely associated with ascidians not surviving on exposed sites where strong solar radiation damages the animals (Bingham and Reyns, 1999; Bingham and Reizel, 2000) or having more spicules and/or UV-absorbing substances for light protection (e.g., Hirose et al., 2006). Ascidian larvae exhibit thigmotactic behavior (Rudolf et al., 2019). On substrates, larvae explore and touch the surface with the tips of their adhesive papillae (Zeng et al., 2019a). Once ascidian larvae adhere to the substrate via adhesive substances secreted from their adhesion papillae, they cannot leave the substrate again. Therefore, substrate selection is crucial for ascidian survival. On the other hand, biofouling of the sessile organisms causes economic damages for fishery, port facilities, and other human activity as well as environmental problems. Therefore, controlling larval settlement has emerged as a critical issue that requires attention (e.g., Bannister et al., 2019). The use of toxic substances to reduce settlement can potentially result in environmental pollution (e.g., Cima and Varello, 2021; Tokur and Aksoy, 2023). If controlling settlement can be attained through the substrate preference of the larvae, this could be a more environmentally friendly approach for combating biofouling. Larval substrate preferences have been studied in some sessile animals, focusing on the properties of the substrate surface, such as surface structures and wettability

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(reviewed in Rittschof et al., 1998; Brady and Singer, 2000; Scardino and de Nys, 2011; Aldred and Clare, 2014; Hirose and Sensui, 2021).

The nano-scale nipple array, often referred to as the moth-eye structure, is an array of protrusions that are 100 nm or less in height and found on the surface of various metazoan taxa. This structure, first described in a nocturnal moth's compound eye, forms a gradient of refractivity, leading to a reduction in light reflectance, known as the moth-eye effect (Bernhard, 1967). Although nano-scale nipple arrays have been reported in marine invertebrates such as tunicates (Hirose et al., 1997; 1999), echinoderms (Holland and Nealson, 1978), annelids (Hausen, 2005), parasitic copepods (Hirose and Uyeno, 2014), and entoprocts (Iseto and Hirose, 2010), these nanostructures in phylogenetically distant taxa are thought to have evolved convergently because of differences in the histological organization of integumentary tissues. Nanoscale nipple arrays also reduce light reflection in water, although this effect is less significant than that in terrestrial environments because of the relatively small differences in the refractive indices between seawater and animal tissues (Kakiuchida et al., 2017). Furthermore, this nano-structure is considered to be a multifunctional one. Employing synthetic materials that imitate this structure has demonstrated that nipple arrays can decrease the surface adsorption and adhesion forces (Uesugi et al., 2022), reduce bubble attachment (Hirose et al., 2013), and suppress immunocyte activity (Ballarin et al., 2015). Interestingly, ascidian larvae prefer a flat surface to the nano-scale nipple array for settlement (Hirose and Sensui, 2019), suggesting that the larvae may sense nano-scale roughness on the substrate surface directly or indirectly. The ascidian larvae employ adhesive papillae to explore the surface of the substrate and then secrete adhesive material for settlement from the tips of the papillae, which are also sensory organs with various senses (reviewed in Pennati and Rothbaecher, 2015). To clarify whether ascidian larvae show different preferences for surface nano-structures of different sizes, we performed a substrate selection assay for test plates made from the same materials. For this assay, we fabricated three types of periodic nano-folds with different heights and pitches.

## MATERIALS AND METHODS

### Animals

Mature individuals of the ascidian *Phallusia philippinensis* were collected by hand at Yonabaru Marina, which is located on the east coast of Okinawajima Island (Japan). The individuals were temporarily reared at approximately 25°C in an aquarium until further use.

### Fabrication of nanoimprinted plates

Three types of periodic nano-folds with different sizes were prepared for the substrate selection assay because the fabrication of nano-scale nipples or pillars is practically difficult owing to technical and facility restraints. They were replicated onto a transparent polymer according to the procedures described by Sakai et al. (2019). Briefly, the surface-relief structure was fabricated on a photosensitive azobenzene polymer film (poly-orange tom-1, Tri Chemical Laboratories) spin-coated on a glass plate (S-1111, Matsunami). Holographic surface-relief gratings were created on the polymer film using two-beam interference exposure with a circularly polarized diode pumped solid state (DPSS) laser (Samba, Cobolt) at 532 nm. The periods of the interference fringes were set to 600, 1000, and 2000 nm by adjusting the angle between two-beam. Negative replicas of the grating structure were then made on transparent thermoset silicone rubbers (ELASTOSIL RT601 A/B, Wacker Asahikasei Silicone). The thermally cured silicone rubbers were used as molds for UV nanoimprinting of the periodic nano-folds. The transparent silicon mold was placed on a glass plate coated with liquid UV-curable resin (NOA61, Norland Products), and the resin was cured by UV irradiation. The surface structures of the UV-imprinted plates were observed using atomic force microscopy (AFM) (Nanocute, SII Nanotechnology). The nanoimprinted plates were cut into 12.5 × 16.5 mm with a glass cutter. We prepared four types of plates with different height of folds and pitch of the folds. They were Flat (no folds), Small (120 nm in height, 600 nm in pitch), Medium (200 nm in height, 1000 nm in pitch), and Large (400 nm in height, 2000 nm in pitch) (Fig. 1). The water wettability on the plates was 80°–90° in contact angle, and considerable differences in wettability were not observed with the size of the nanostructures.

### Substrate selection assay (Fig. 2)

This assay was essentially the same as that described by Sensui and Hirose (2020). Seven *P. philippinensis* individuals were dissected, and eggs and sperm were collected from the oviduct and sperm ducts in their hermaphroditic bodies. Sperm were briefly incubated in high-pH artificial seawater (pH 9.0) to activate their motility. The eggs were inseminated with activated sperm from another individual and rinsed with artificial seawater 15 min after insemination. The larvae hatched 12–13 h after insemination, following incubation at 24–25°C.

Each of the four types of test plates (Flat, Small, Medium, Large; 12.5 × 16.5 mm) were fixed on the outer bottom of a plastic dish (53 mm diameter) with a double-coated adhesive tape, and the remaining plastic surfaces were masked with a super-hydrophilic film (SH2CLHF, 3M) to prevent larval settlement on the dish surface other than the test plates. The inner surface of a glass dish (inner diameter, 55 mm) was coated with 1.5% agar to prevent larval set-

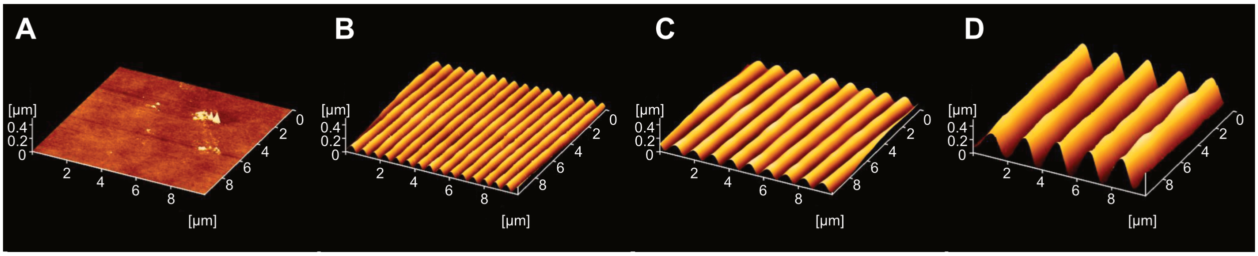

**Fig. 1.** AFM images of periodic nano-folds on test plates. **(A)** Flat (no nano-folds). **(B)** Small (120 nm in height, 600 nm in pitch). **(C)** Medium (200 nm in height, 1000 nm in pitch). **(D)** Large (400 nm in height, 2000 nm in pitch). Note that the scale differed between the height and horizontal directions.

tlement, and 15 mL of artificial seawater containing 500–2000 freshly hatched larvae was added to the dish. The plastic dish holding the four test plates was then floated on the seawater in this glass dish. The dishes were placed inside a tin box to shield them from light and reduce the evaporation of seawater, and incubated for 24 h at 24–25°C. After incubation, we photographed each plate and counted the number of larvae that settled on each test plate from digital images. A marginal zone of 0.3 mm width on each test plate was excluded from the count to avoid irregular settlement due to the edge effect. Nine sets of assays were performed simultaneously.

Statistical calculations were performed using R software (R Core Team, 2024). The difference in the ratio of larval settlement among the test plates was tested using one-way ANOVA. Manly's resource selection index uses the ratio of usage to availability of resources and evaluates selectivity using Bonferroni confidence intervals based on a chi-square test (Manly et al., 2002). Selection indices for each substrate were calculated to test for significant preference for settlement using the Resource Selection Program (Okamura et al., 2004) for R, and pairwise comparison of the indices was performed with Bonferroni correction.

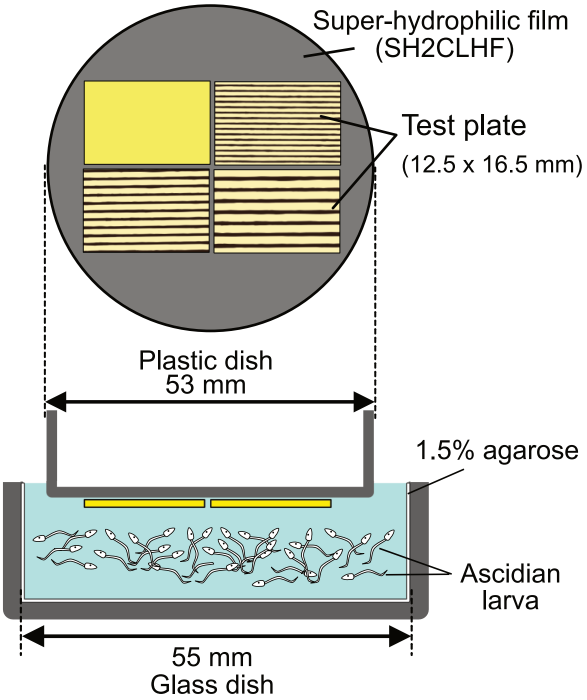

**Fig. 2.** Schematic representation of the substrate selection assay.

## RESULTS AND DISCUSSION

The larvae of *P. philippinensis* settled on all the test plates in all nine sets of assays, whereas the number of settlements varied among the plates. As the number of larvae placed in the glass dish differed among the sets, the total number of settlements in each set varied from 330 to 1356. Therefore, we compared larval settlement as the ratio of larval settlement on each test plate to total settlement in each set (Fig. 3, and see Supplementary Table S1). The ratios varied considerably among the sets of the assay, and one-way ANOVA test did not support significant differences among the test plates ($P$ = 0.096). The Manly's resource selection indices and the Bonferroni confidential intervals of each test plate (index; interval) were as follows: Flat (1.16; 1.06–1.26), Small (0.79; 0.70–0.87), Medium (1.08; 0.98–1.17), and Large (0.98; 0.89–1.07). Accordingly, significant preferences were supported for Flat (positive preference) and Small (negative preference). Pairwise comparison of the resource selection indices with Bonferroni correction supported that Small was significantly less selected than any other substrate, and Flat was significantly more selected than Small and Large ($P$ < 0.01) (Fig. 4).

The larvae of *P. philippinensis* appeared to prefer flat surfaces over surfaces with periodic nano-folds for settlement and showed a negative preference for the folds of 120-nm height. Owing to the difficulty in fabricating nano-scale nipple arrays of different sizes, we fabricated and used periodic nano-folds rather than nipple arrays in the assays. As observed in the negative preference for nano-scale-nipple arrays (Hirose and Sensui, 2019), substrate preference was also observed in the periodic nano-folds, indicating that the

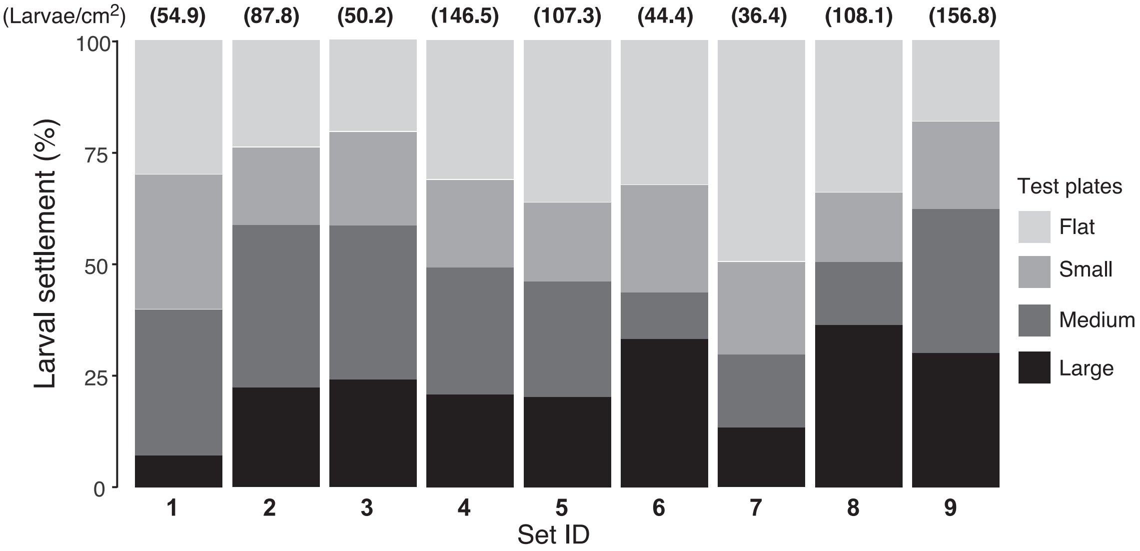

**Fig. 3.** Ratio of larval settlement on each substrate to total settlement (%) in each of the nine sets of assays. Averages of larval settlement per square centimeter in each set are shown in parentheses above the bars.

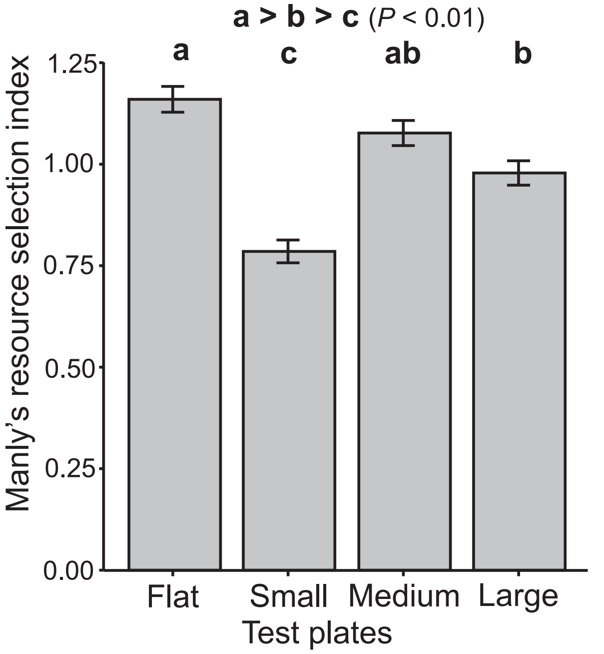

**Fig. 4.** Comparison of Manly's resource selection indices among the test plates. Error bars indicate the standard error. The alphabetical symbols indicate significant differences in the pairwise test with Bonferroni correction ($P < 0.01$).

nano-folds can be functional nano-structures. Periodic nano-folds have also been found on animal body surfaces, such as the colonial ascidian *Clavelina* spp. (Hirose et al., 1990; Sakai et al., 2019) with a presumed enhancement in the reduction of bubble adhesion and light reflection (Sakai et al., 2019).

Significant differences in the selection indices of nano-folds of different sizes indicate that larvae can not only detect the presence or absence of nanostructures, but also distinguish between nanostructures of different sizes. The three types of periodic nano-folds fabricated herein (i.e., Small, Medium, and Large) were geometrically similar (differing only in size). The larvae may directly sense these differences in size or indirectly sense the difference in surface properties due to the size of the nano-folds. On the substrate for settlement, swimming larvae often touch the substrate surface with the tips of their adhesive papillae as if examining the surface properties. Microvilli or cellular processes are extended from the tip of the adhesion papillae of larvae (e.g., Dolcemascolo et al., 2009), and they likely sense the properties of the substrate surface. Adhesive papillae have both sensory and secretory functions (Pennati and Rothbaecher, 2015) and each papilla projects nerve bundles to the cerebral ganglion of the larva (Imai and Meinertzhagen, 2007; Zeng et al., 2019b). In ascidian larvae, polymodal sensory perception is involved in adhesion and metamorphosis on the substrate (Hoyer et al., 2024), and the larvae likely use various types of sensory information for substrate selection. However, it remains unclear how larvae recognize and discriminate between surface nanostructures.

Substrate material and surface roughness are known to affect biofouling. The formation of centimeter-scale topographic complexity by the addition of mussel and oyster shells to concrete resulted in drastic changes in the species composition of benthic communities (Queiroz et al., 2024). Chase et al. (2016) demonstrated that substrate selection by ascidian larvae was influenced by both the species of ascidian and the material of the substrate, and they suggested that the micro-scale roughness of the surface might play a role in determining preference. On the other hand, Groppelli et al. (2003) found that the preference of ascidian larvae varied based on the mineral content of the substrate but did not observe significant differences in preference based on the micro-scale roughness of the surface. In the bivalve *Mytilus galloprovincialis*, the size of the micro-scale surface structures has a significant effect on larval settlement, with low settlement rates on flat and 10–20 μm high structures and significantly higher settlement rates on structures 40–80 μm and 300–1000 μm in height (Carl et al., 2012). In the present study, ascidian larvae showed a positive preference for smooth surfaces over nanostructured surfaces, but it should be considered that the mechanisms of substrate selection are different on nano- and micro-structured surfaces, as well as in different animals. Although superhydrophobic coatings with nanoscale roughness have been shown to have effective anti-fouling properties against a broad spectrum of fouling organisms (Scardino et al., 2009), considering that this property is due to their extraordinary wettability, it is difficult to compare them with those in the present study. Since the larvae had a significantly negative preference for the Small (height, 120 nm; pitch 600 nm) for settlement among the parallel nano-folds tested here, the size is likely important for the surface properties provided by nano-structures. Although nipple heights are 200 nm or more in some terrestrial insects (e.g., Spalding et al., 2019), the nano-scale nipple arrays found on aquatic metazoans are usually approximately 100 nm or less in height, suggesting a functional constraint for the size of nano-structures. In other words, to reduce the fouling of settlers with a negative preference for nanostructures of approximately 100 nm (or less) in height, organisms may have nano-structures of similar sizes on their body surfaces to reduce biofouling.

Substrate preference assays using periodic nano-folds revealed that ascidian larvae recognize nano-scale differences and show significantly negative selectivity, especially for nano-folds 120 nm in height. This is the first report to demonstrate that planktonic larvae of sessile animals recognize differences in nanostructure size during substrate selection for settlement. It is uncertain whether larvae can directly sense nano-scale differences in dimensions or whether they can sense differences in the physical properties of the substrate surface owing to nano-structures. In either case, understanding why larvae discriminate between nano-differences and select for settlement is a crucial factor in effectively managing ascidian biofouling without using harmful methods.

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## COMPETING INTERESTS

The authors have no competing interest to declare.

## AUTHOR CONTRIBUTIONS

DS fabricated the test plates emerging nano-fold, and NS carried out substrate selection assays. EH designed the study and drafted the manuscript. All authors approved the final manuscript for publication.

## SUPPLEMENTARY MATERIALS

Supplementary material for this article is available online. (URL: https://doi.org/10.2108/zs240066)

**Supplementary Table S1.** Number of larval settlements/cm$^2$ on each test plate and ratio of larval settlement on each test plate to the total settlements (%).

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