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Evaluation of species-specificity in barnacle waterborne settlement pheromones

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Abstract

Larval settlement is an important process that drives population and community dynamics of marine invertebrates. Barnacles are frequently used to investigate settlement mechanisms of marine invertebrate larvae. Adult barnacles induce settlement of conspecific larvae nearby which ultimately facilitates copulation with neighbouring individuals. A significant factor involved in the larval induction process is the proteinaceous waterborne settlement pheromone (WSP), which is purified from adult barnacles. A previous study suggested that the concentration of WSP informs barnacle cyprid larvae about the abundance of adult barnacles in the environment nearby but it is unclear whether WSP works in a species-specific or non-species-specific manner. In this study, we conducted settlement assays using recombinant WSPs and cyprids of two congenic barnacle species, *Amphibalanus amphitrite* and *A. improvisus*, to investigate the species specificity of WSPs. We found that *A. amphitrite* and *A. improvisus* cyprids responded similarly to con-specific WSPs as to allo-specific WSP stimuli indicating that WSPs are not species-specific. Our findings suggest that cyprids approach potential settlement sites using WSPs, non-species-specific settlement pheromones, before performing a closer search of the substratum using a species-specific pheromone.

Introduction

It is widely observed that marine invertebrates undergo dramatic transitions from their planktonic phase to benthic phases. During this process, the planktonic larvae of these organisms actively approach nearby adult habitats to settle. To find suitable sites for settlement, these larvae rely on various physico-chemical and biological cues including settlement pheromones (Pawlik, 1992; Hodin *et al.*, 2018; Bilodeau and Hay, 2021).

Barnacles, often used as organisms to investigate settlement mechanisms, exhibit hermaphroditism and engage in gamete exchange with nearby individuals. The planktonic phase of their life cycle begins with the release of nauplius larvae from an adult, which then disperse in the water column. These larvae undergo metamorphosis into cyprid larvae, equipped with chemo/mechano-sensory setae on a pair of appendages that are responsible for exploring potential substrates (Moyse, 1995; Bielecki *et al.*, 2009; Maruzzo *et al.*, 2011; Abramova *et al.*, 2019*a*). Upon detecting a suitable site, cyprids settle and transition to the benthic phase. If unsuitable, they continue to swim and explore other locations.

Adult barnacles induce nearby settlement of conspecific cyprids to facilitate reproduction. This process involves 3–5 kDa (Rittschof, 1985, 1990, 1993; Rittschof and Cohen, 2004) or <500 Da (Clare and Matsumura, 2000) peptides released into the seawater by adult barnacles. In addition, di- and tri-peptides with arginine or lysine at the N-terminus have been reported to induce settlement (Tegtmeyer and Rittschof, 1988), although the effectiveness of these peptides in inducing settlement is still a matter of debate (Clare and Yamazaki, 2000). Recently, adenosine was also reported to induce settlement (Wu *et al.*, 2024). Two proteinaceous settlement pheromones, Settlement-Inducing Protein Complex (SIPC) (Matsumura *et al.*, 1998*b*) and waterborne settlement pheromone (WSP) (Endo *et al.*, 2009), have also been identified. SIPC is presumed to be adsorbed on substrate surfaces and adult barnacle shells and it functions in the vicinity of adult barnacles (Matsumura *et al.*, 1998*b*; Clare and Matsumura, 2000; Dreanno *et al.*, 2006*b*; Clare, 2010; Petrone *et al.*, 2015). Cyprids leave SIPC footprints on the substratum during exploration, promoting larva-larva interactions (Clare *et al.*, 1994; Matsumura *et al.*, 1998*c*; Dreanno *et al.*, 2006*a*). Furthermore, SIPC functions in a concentration-dependent manner, with settlement being induced at lower concentrations and inhibited at higher concentrations (Kotsiri *et al.*, 2018). This suggests that cyprids are capable of assessing the density of barnacles on a substrate and will not settle if the site is overcrowded (Kotsiri *et al.*, 2018). Understanding the mechanisms by which conspecific cues guide larvae to settlement sites can provide valuable insights into population and community dynamics of marine invertebrates (Jenkins, 2005; Zimmer *et al.*, 2016).

Another pheromone, WSP, can diffuse into the seawater when embedded in agarose gel suggesting that it attracts cyprids on a larger spatial scale compared to SIPC (Endo *et al.*, 2009; Clare, 2010). While field-based studies on WSP have not been reported yet, it is

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suggested that WSPs work within tens of centimetres from adult habitats (Elbourne and Clare, 2010). Moreover, recent studies identified WSP in the adhesive layer of adult barnacles supporting that WSP is released into the environment (So *et al.*, 2017; Schultzhaus *et al.*, 2019, 2020, 2021).

Transcriptome and proteome research have shown that there are multiple homologues of WSP (So *et al.*, 2017; Schultzhaus *et al.*, 2019, 2020, 2021; Abramova *et al.*, 2019*b*). Of these homologues in *A. amphitrite*, the 'original WSP' (hereafter referred to as 'WSP'), as reported by Endo *et al.* (2009), is most abundant in the cement of adult barnacles (So *et al.*, 2017; Schultzhaus *et al.*, 2019, 2020). These findings also suggest that the concentration of WSP is an important information for cyprids. According to Kitade *et al.* (2022), low concentrations of barnacle WSP decrease the likelihood of cyprids settling, whereas high concentrations have the opposite effect. Weak WSP signals may indicate to cyprids that adult barnacles are not abundant where they are checking the substrate, leading them to prolong their larval phase (Kitade *et al.*, 2022). However, the species specificity of WSP has not been thoroughly investigated. Therefore, we conducted settlement assays using recombinant WSPs and cyprids of two barnacle species, *A. amphitrite* and *A. improvisus*, to investigate the species specificity of WSPs.

Materials and Methods

Recombinant proteins of waterborne settlement pheromones

WSPs of two barnacle species, *Amphibalanus amphitrite* and *A. improvisus*, were synthesized using an *Escherichia coli* expression system. The *A. amphitrite* recombinant WSP used in this study is identical to the one used in a previous paper, which confirmed that the recombinant *A. amphitrite* WSP showed the same activity in induction of larval settlement as native WSP purified from adult barnacles (Kitade *et al.*, 2022). The purity of the recombinant *A. amphitrite* WSP was > 90% and showed a single main band on SDS-PAGE (Kitade *et al.*, 2022). The complete cDNA sequences of *A. improvisus* WSP were reported by Abramova *et al.* (2019*b*) and the recombinant *A. improvisus* WSP with a molecular mass of 28.4 kDa was synthesized using the method described by Kitade *et al.* (2022). Briefly, DNA fragments of the *A. improvisus* WSP (Accession Number MK275628) that lacked its signal peptide were synthesized with a NdeI site (5′-CATATG-3′) and a His-Tag (5′-CACCATCATCATCATCAT-3′) at the 5′ end and a Hind III site (5′-AAGCTT-3′) added at the 3′ end. It was then ligated into pET30a vectors and transformed into *E. coli*. After IPTG (Isopropyl β-D-1-thiogalactopyranoside) induction, the protein solution was obtained from the *E. coli* pellet and the recombinant WSP was purified using His Trap. The purified recombinant WSP was dialyzed against 50 mM Tris-HCl buffer (pH 7.5). The purity of the protein was checked through SDS-PAGE under denaturing and reducing condition with a reducing agent. The purity of the recombinant *A. improvisus* WSP was > 80% and showed a single main band on SDS-PAGE (Figure S1).

Adult barnacles and larval culture

Adults of *A. amphitrite* and *A. improvisus* were collected in Kobe, Japan from November 2021 to January 2022. Nauplii released from the adults were cultured in filtered seawater (FSW) containing penicillin/streptomycin (FUJIFILM Wako Pure Chemical Co. Osaka, Japan) to suppress the growth of bacteria and fungi. Nauplii were fed with a diatom *Chaetoceros calcitrans* at 22°C under a 12 h:12 h light cycle and metamorphosed into cyprids after 5–7 days. Only cyprids swimming towards the light source were collected, rinsed with FSW, and kept at 5°C for 1 day.

Larval settlement assays

Larval settlement-inducing activities were determined in single-larva settlement assays using 48-well polystyrene plates. Single cyprids of *A. amphitrite* or *A. improvisus* were placed in the wells. Then, 80% FSW containing allo-specific recombinant WSPs was added at concentrations of 1, 10, and 100 nmol l^{-1} for *A. amphitrite*, and both con- and allo-specific recombinant WSPs were added at the same concentrations for *A. improvisus*, all at a final volume of 600 µl. As a control, 50 mM Tris-HCl buffer (pH 7.5) was added. The cyprids was incubated at 22°C in the dark. *Amphibalanus improvisus* cyprids took a longer period to settle than *A. amphirite* and the number of settled cyprids was counted after 24 h for *A. amphitrite* and 72 h for *A. improvisus*. Each treatment had approximately 30 to 50 replicates from two to four batches of larvae. The settlement response of *A. amphitrite* cyprids to conspecific WSP has reported by Kitade *et al.* (2022).

Data analyses

A binomial Generalized Linear Mixed Model (GLMM) was performed to assess the effect of concentration of WSPs on cyprid response among treatments with larval batch as a random effect. In the models, concentrations of WSPs were treated as categorical data. All statistical analyses were performed using R 4.0.4 (R Core Team, 2021). GLMM and post-hoc tests were performed using lem4 (Bates *et al.*, 2015) and the Anova function of the R package car 3.1.2 (Fox and Weisberg, 2019), respectively. The Holm-Bonferroni method (Holm, 1979) was used to adjust *P*-values.

To evaluate the species specificity of WSPs, we used binomial regression models using a series of models incorporating concentrations of WSPs, species of WSPs, and interactions between these factors with larval batch as a random factor to predict the settlement probabilities for each cyprid species (full model: (Settlement ~ Concentrations_of_WSPs × Species_of_WSPs + (1 | Batch))). For each model, Akaike Information Criterion (AIC) weighting were calculated using the R package MuMIn 1.47.5 (Bartoń, 2023). The model with the highest AIC weighting was selected as the best-fit model. For both *A. amphitrite* and *A. improvisus* cyprids, models with concentrations of WSPs and concentrations of WSPs + species of WSPs were selected as the best-fit and second best-fit models, respectively (see Results section). Then these models were compared using the Anova function of the R package car 3.1.2 (Fox and Weisberg, 2019). Data on settlement of *A. amphitrite* cyprids in response to the conspecific WSP were obtained from Kitade *et al.* (2022). All the settlement assay data used in this study were shown in Tables S1–S4.

Results

Amphibalanus amphitrite cyprids showed a non-linear response to *A. improvisus* WSP concentration (Figure 1B, Figure S2) which is similar to the pattern observed in response to conspecific WSP (Figure 1A; Kitade *et al.*, 2022). Binomial GLMM analysis indicated that the probability of settlement was significantly lower in 1 nmol l^{-1} WSP than in controls or in 10 nmol l^{-1} WSP ($P < 0.05$). The probability of settlement was significantly higher in the 100 nmol l^{-1} allo-specific WSP treatment compared to the other concentrations in *A. amphitrite* cyprids (Figure 1B, $P < 0.05$). *Amphibalanus improvisus* cyprids showed higher settlement in the 100 nmol l^{-1} treatment than in the other treatments for both conspecific and allo-specific WSPs (Figure 2, Figure S3–S4, $P < 0.05$). Note that the settlement probability in the control treatment was lower in *A. improvisus* cyprids (about 5–10%) than in *A. amphitrite* cyprids (about 25–30%). For *A. amphitrite* cyprids, the models with concentrations of WSPs

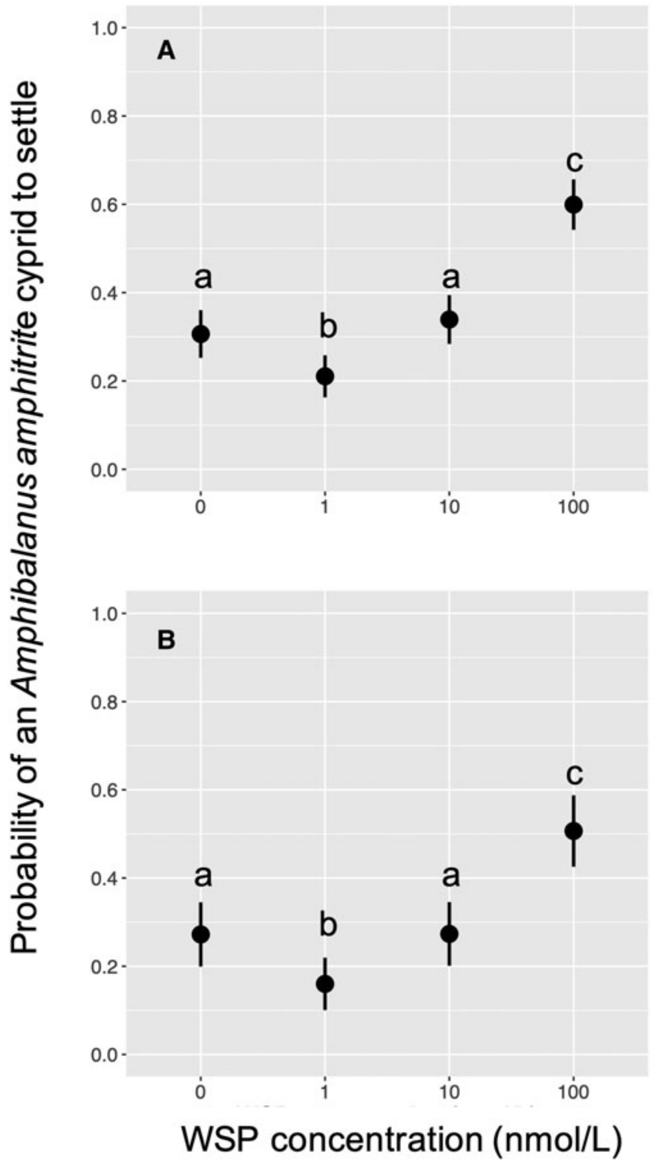

Figure 1. Probability of an *Amphibalanus amphitrite* cyprid to settle under the stimulus of WSPs of (A) *A. amphitrite* and (B) *A. improvisus*. Results of pooled data from all the larval batches are shown. Results of each larval batch is shown in Figure S1. Bars indicate 95% confidence intervals. Different letters indicate significant differences ($P < 0.05$). (A) is modified from Kitade *et al.* (2022) and published with open access under the Creative Commons CC-BY license.

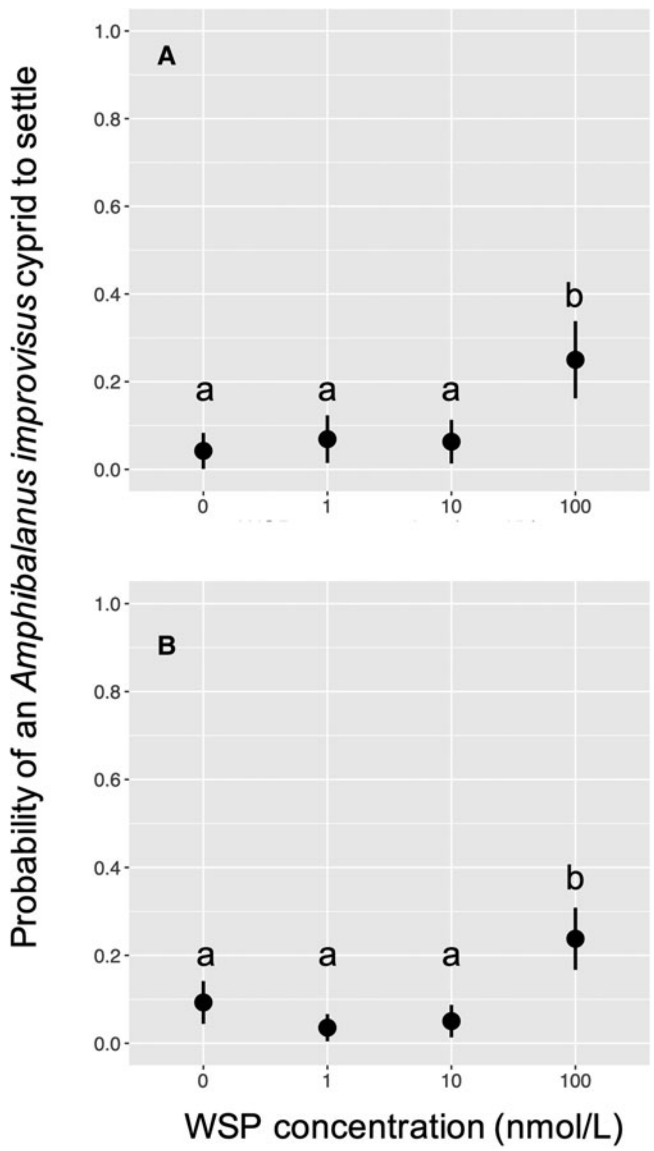

Figure 2. Probability of an *Amphibalanus improvisus* cyprid to settle under the stimulus of WSPs of (A) *A. improvisus* and (B) *A. amphitrite*. Results of pooled data from all the larval batches are shown. Results of each larval batch is shown in Figure S2–S3. Bars indicate 95% confidence intervals. Different letters indicate significant differences ($P < 0.05$).

(AIC weight 0.519) and concentrations of WSPs + species of WSPs (AIC weight 0.453) were selected as the best-fit and second best-fit models, respectively (Table 1). Similarly, for *A. improvisus* cyprids, models with concentrations of WSPs (AIC weight 0.646) and concentrations of WSPs + species of WSPs (AIC weight 0.262) were selected as the best-fit and second best-fit models, respectively (Table 1). No significant differences were detected between the best-fit and second best-fit models in cyprids of *A. amphitrite* ($P = 0.189$) and *A. improvisus* ($P = 0.656$).

Table 1. Akaike Information Criterion (AIC) weights for binomial regression models predicting settlement probabilities of cyprid of *Amphibalanus amphitrite* and *A. improvisus* using concentrations of WSPs, species of WSPs, and interactions between these factors, with larval batches as a random factor

	AIC weight	
Model	*A. amphitrite*	*A. improvisus*
Concentrations of WSPs	0.519	0.646
Concentrations of WSPs + Species of WSPs	0.453	0.262
Interaction between concentrations and species of WSPs	0.028	0.092
Null	0.000	0.000
Species of WSPs	0.000	0.000

Settlement data of *A. amphitrite* cyprids in response to the conspecific WSP were obtained from Kitade *et al.* (2022).

Discussion

Our previous study found that low concentrations of *A. amphitrite* WSP decreased the probability of settlement of conspecific cyprids, whereas high concentrations had the opposite effect (Kitade *et al.*, 2022). The study suggested that weak WSPs inform cyprids that adult barnacles are not abundant where they are checking the substrate, inducing them to extend their larval

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phase (Kitade *et al.*, 2022). In the present study, significant settlement suppression under the condition of low *A. improvisus* WSP concentration was not detected in the conspecific cyprids (Figure 2). This may be because of the lower settlement probability in the control treatment in *A. improvisus* cyprids than in *A. amphitrite* cyprids, resulting in difficulty to detect the effect of weak WSPs. Overall, the probability of settlement tended to be higher in *A. amphitrite* than in *A. improvisus*, although the reason for this remains unclear.

Amphibalanus amphitrite and *A. improvisus* cyprids responded similarly to allo-specific WSPs as to con-specific WSP stimuli. Furthermore, models incorporating only concentrations of WSPs were selected as best-fit models in both species (Table 1), supporting that WSPs are not species-specific. Amino acid sequences of *A. amphitrite* and *A. improvisus* WSPs show 91% similarity and no obvious differences were observed between them (Abramova *et al.*, 2019*b*). In another proteinaceous settlement pheromone, SIPC, sugar chains are important for the settlement-inducing activity and species specificity (Matsumura *et al.*, 1998*a*; Pagett *et al.*, 2012; Yorisue *et al.*, 2012). For instance, it was revealed that the larval settlement-inducing activity of SIPC is inhibited by lentil lectin, which binds to sugar chains, suggesting that sugar chains are functionally important in SIPC (Matsumura *et al.*, 1998*a*). In contrast, treatments with lentil lectin had no effect on the function of native WSP (Endo *et al.*, 2009). Our previous paper confirmed that the recombinant *A. amphitrite* WSP made with the *E. coli* expression system shows the same activity in inducing larval settlement as native WSP protein purified from adult barnacles (Kitade *et al.*, 2022). These results suggest that glycosylation is not important for the settlement-inducing activity of WSP. However, additional studies using natural WSPs or recombinants from animal cell expression systems are needed to understand the effects of glycosylation on the settlement-inducing activity and species specificity of WSPs.

Amphibalanus amphitrite and *A. improvisus* were introduced to Japanese waters in the 20th century (Iwasaki *et al.*, 2004). Currently, these two species coexist and are commonly found along the Japanese coasts. The origin of *A. amphitrite* is suggested to be Indo-Pacific (Carlton *et al.*, 2011), and that of *A. improvisus* is Atlantic (Carlton *et al.*, 2011; Wrange *et al.*, 2016). However, *A. amphitrite* cyprids are suggested to distinguish between conspecifics and allospecifics, including *A. improvisus*, by means of sensing another proteinaceous settlement pheromone, SIPC (Dreanno *et al.*, 2007). This is further supported by a genetic study which showed that SIPC contains variable regions among these species, which are suggested to contribute to the species specificity (Yorisue *et al.*, 2012). Additionally, there is evidence that *A. amphitrite* cyprids are capable of distinguishing between conspecifics and *Semibalanus balanoides* which is distributed in northwestern Europe and North America and vice versa (Matsumura *et al.*, 2000). These reports suggest that cyprids responded to allo-specific WSPs not because WSP speciation did not occur between geographically separated species. Therefore, our results suggest that WSPs are not species-specific.

Peptides obtained from seawater conditioned by *S. balanoides* have been shown to induce settlement of *A. amphitrite* cyprids (Rittschof, 1985), suggesting that waterborne peptide pheromones may not be species-specific. Adenosine has also recently been identified as a pheromone (Wu *et al.*, 2024), but it is unclear how these molecules act in combination. Cyprids are suggested to be guided to potential settlement substrates by sensing WSPs and/or peptide pheromones in a non-species-specific manner, possibly within tens of centimetres from adult habitats (Elbourne and Clare, 2010). However, Abramova *et al.* (2019*b*) reported the existence of WSP homologues and pointed out the possibility that the blend ratio may determine species specificity. Further studies are needed to test this hypothesis. Cyprids, then, closely examine the substratum using SIPCs adsorbed on the substratum or adult shells (Matsumura *et al.*, 1998*b*; Clare, 2010; Petrone *et al.*, 2015) in a species-specific manner (Kato-Yoshinaga *et al.*, 2000; Dreanno *et al.*, 2007; Yorisue *et al.*, 2012) to determine the ideal settlement position. Cyprids themselves also secrete SIPC from their antennules to leave footprints for larva-larva interactions (Clare *et al.*, 1994; Matsumura *et al.*, 1998*c*; Dreanno *et al.*, 2006*a*). Additionally, SIPC can act as an avoidance cue when its concentration is high, indicating overcrowding (Kotsiri *et al.*, 2018). We suggest that cyprids approach and select their settlement sites by utilizing a combination of species-specific and non-species-specific pheromones across different spatial scales. Thus, the use of species-specific and non-specific settlement pheromones at different spatial scales may facilitate the efficient formation of breeding populations. Many barnacles are known to be exotic species in coastal environments around the world (Iwasaki *et al.*, 2004; Carlton *et al.*, 2011), and employing such spatially scale-specific sessile pheromones may enable them to efficiently establish populations in introduced environments. The species pairs used in this study *A. amphitrite* and *A. improvisus*, are congenic and both inhabit brackish intertidal zones. It remains unclear whether cyprids can distinguish WSPs of species from phylogenetically distant or other habitats.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S002531542400095X.

Data. All data on settlement assays are included in the Supporting Information.

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Author contributions. All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Shiori Kitade, and Takefumi Yorisue. The first draft of the manuscript was written by Shiori Kitade and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Conflict of interest. None.

Ethical standards. Not applicable.

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