Uptake and Persistence of Homologous and Heterologous Zooxanthellae in the Temperate Sea Anemone *Cereus pedunculatus* (Pennant)

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Abstract. The uptake and persistence of symbiotic dinoflagellates (zooxanthellae) were measured in the temperate sea anemone *Cereus pedunculatus* (Pennant). Aposymbiotic specimens of *C. pedunculatus* were inoculated with zooxanthellae freshly isolated from a range of temperate and subtropical Anthozoa. Each inoculate consisted of zooxanthellae from a single host species and was either homologous (zooxanthellae from a host of the same species as the one being inoculated) or heterologous (from a host of a different species than the one being inoculated). The densities of zooxanthellae in host tissues were determined at regular intervals. *C. pedunculatus* took up homologous and heterologous zooxanthellae to similar degrees, except for zooxanthellae from the temperate *Anthopleura ballii*, which were taken up to a lesser extent. The densities of all zooxanthellae declined between 4 hours and 4 days after uptake, indicating that zooxanthellae were expelled, digested, or both during this period. The densities of all zooxanthellae increased between 2 and 8 weeks after inoculation, indicating zooxanthella growth. Over the entire 8-week period after uptake, densities of homologous zooxanthellae were always greater than those of heterologous zooxanthellae. Between 8 and 36 weeks after infection, densities of homologous zooxanthellae declined markedly and densities of some heterologous zooxanthellae increased further, resulting in homologous and heterologous zooxanthella densities being the same at 36 weeks. These densities were the same as those in naturally infected *C. pedunculatus* of similar size. The results suggest that zooxanthellae from a range of host species and environments can establish symbioses with *C. pedunculatus* and that, over long periods under laboratory conditions, heterologous zooxanthellae may populate *C. pedunculatus* to the same extent as homologous zooxanthellae.

Introduction

Many benthic marine Cnidaria possess endosymbiotic dinoflagellates (zooxanthellae) of the genus *Symbiodinium*. These Cnidaria may lose zooxanthellae entirely (i.e., become aposymbiotic) when under environmental stress (Williams and Bunkley-Williams, 1990; Glynn, 1991) or when zooxanthellae are not inherited maternally (Trench, 1987; Shick 1991). Consequently, they may be susceptible to reinfection and colonization by zooxanthellae released from a range of host species (Schoenberg and Trench, 1980c; Rowan and Powers, 1991b; Buddemeier and Fautin, 1993). Despite this susceptibility and the high diversity of hosts and symbionts (Trench, 1987; Rowan and Powers, 1991b; Trench, 1993; McNally et al., 1994), many species of Cnidaria have been reported to harbor just one strain or species of *Symbiodinium* (Schoenberg and Trench, 1980a, b; Rowan and Powers, 1991a, b; Trench, 1993). Other hosts have been found to contain more than one strain or species of zooxanthella, but even in these cases the algae are the same across the host’s geographical range (Trench and Winsor, 1987; Rowan and Powers, 1991b; Rowan and Knowlton, 1995).

The uptake and persistence of zooxanthellae following
inoculation of aposymbiotic hosts has been investigated in a range of tropical marine Cnidaria (Kinzie, 1974; Kinzie and Chee, 1979; Schoenberg and Trench, 1980c; Trench, 1981; Trench et al., 1981; Colley and Trench, 1983; Fitt and Trench, 1983a, b; Berner et al., 1993). The zooxanthellae were either homologous (from a host of the same species of cnidian as the one being inoculated) or heterologous (from a host of a different species than the one being inoculated). In contrast, similar work has been performed using just one species of temperate marine Cnidaria, the North American sea anemone Anthopleura elegantissima (Trench, 1969, cited in Trench, 1971; Muller-Parker, 1996; Weis and Levine, 1996).

The temperate sea anemone Cereus pedunculatus (Pennant) (family Sagartiidae) is locally abundant around the south and west coasts of Europe, where it is found partially buried in sand and mud from the midshore to a depth of 25 m (Manuel, 1981). C. pedunculatus may reproduce by oviparity, but frequently it is hermaphroditic or parthenogenetic and viviparous (Rossi, 1975; Schäfer, 1981; Shick, 1991). The zooxanthellae of C. pedunculatus are located within the host’s endodermal cells and have been identified as Symbiodinium sp. (Davy et al., in press). Oocytes may contain up to 300 zooxanthellae, suggesting that C. pedunculatus acquires its symbionts through maternal inheritance (Turner, pers. obs.). Brooded and recently released juvenile C. pedunculatus always harbor zooxanthellae, but densities in recently released individuals may be less than 10% of those in adult anemones (Darmayati, 1993; Davy et al., 1996). The fact that C. pedunculatus produces offspring that lack a full complement of zooxanthellae means that they may be susceptible to infection by zooxanthellae released from other Anthozoa.

The sea anemones Anthopleura ballii (Cocks) (family Actiniidae) and Anemonia viridis (Forskål) (family Actiniidae), and the zoanthid Isozoanthus sulcatus (Gosse) (family Parazoanthidae) are frequently found in the same localities as C. pedunculatus, and also harbor Symbiodinium sp. (Davy et al., in press). A. ballii and A. viridis regularly expel zooxanthellae in large boluses, as well as in mucous strands (Davy, pers. obs.); it is also probable that I. sulcatus releases zooxanthellae, but this has not been observed. This expulsion suggests that zooxanthellae from a variety of sources are available to infect new hosts.

This research therefore aimed to determine whether heterologous zooxanthellae can infect C. pedunculatus, and to compare the persistence of homologous and heterologous zooxanthellae after infection. From this information the potential for heterologous zooxanthellae to establish a lasting symbiosis with C. pedunculatus in the field could be inferred.

Materials and Methods

Collection and maintenance of symbiotic Anthozoa

The sea anemones Cereus pedunculatus (Pennant), Anemonia viridis (Forskål), and Anthopleura ballii (Cocks), and the zoanthid Isozoanthus sulcatus (Gosse) were collected from Lough Hyne Marine Nature Reserve, Eire (51°29' N; 9°18' W). All four species are exclusively subtidal at this location. The anemones were all collected from a depth of 1 to 3 m, and the zoanthids from 5 to 9 m. Specimens of A. viridis were also collected from the intertidal zone at Shell Island, North Wales (52°47' N; 004°06' W). For comparative purposes, specimens of the subtropical sea anemone Aiptasia pallida (Verrill), cloned from one individual, were obtained from Dr. C. B. Cook, Bermuda Biological Station. The A. pallida had originally been collected from a mangrove root in Walsingham Pond, Bermuda.

Adult Anthozoa were kept in seawater from the Menai Strait, North Wales. They were maintained at 21°C, illuminated at 80 μmol photons m⁻² s⁻¹ on a cycle of 12 h light:12 h dark, and fed twice weekly with Artemia sp. (Bonneville Artemia International Inc.). The light and temperature regimes were comparable to those experienced during a warm summer in Lough Hyne (Turner, 1988, and his pers. obs.).

Preparation of aposymbiotic Anthozoa

Juvenile specimens of C. pedunculatus were squeezed from the enterons of brooding adult anemones and placed in a dark-box to render them aposymbiotic. Seawater from the Menai Strait, at the ambient temperature (10–18°C), flowed through the dark-box continuously. It is unlikely that zooxanthellae were present in this seawater because Anthozoa-zooxanthella associations do not occur locally. In addition, the examination of dark-treated anemones suggested that infections had not occurred under these conditions (see below). The juveniles were collected on two occasions, with the first batch (n = 250) remaining in the dark for more than 3 years and the second batch (n = 120) remaining in the dark for 5 months. After collection, the anemones were fed every 2 months with Artemia sp; the anemones also received planktonic food via the flow-through seawater system. It is likely that the juveniles were genetically related to one another because all adult anemones were collected from the same site and spawning of gametes has not been observed in this population (Davy and Turner, pers. obs.).

Prior to use, 3-year dark-treated anemones (n = 180) with oral disc diameters of about 2 to 3 mm were placed in 100-ml containers filled with filtered (0.45 μm) seawater (FSW). Five anemones were placed in each container.
These anemones were maintained at 21°C, illuminated at 80 μmol photons m⁻² s⁻¹ on a cycle of 12 h light:12 h dark, and fed weekly with Artemia sp. for 2 weeks. The anemones were never observed to change from white to brown over this period and so were assumed to be aposymbiotic. This assumption was checked by squashing a subsample of five anemones (anemones sampled from the same container; n was limited by anemone availability) and examining their tissues under bright-field and epifluorescent illumination using a Leitz Orthoplan microscope. Neither zooxanthellae nor chlorophyll a were detected, confirming that the 3-year dark-treated anemones were aposymbiotic. Therefore, immediately after being maintained in the light for 2 weeks, these anemones were used for monitoring patterns of long-term infection by zooxanthellae. Non-inoculated control polyps used in these experiments remained zooxanthella-free over a period of 36 weeks, reconfirming that the anemones were aposymbiotic.

In contrast, squashes made of 5-month dark-treated polyps (n = 5) before they were placed in the light revealed a small number of residual zooxanthellae (less than 1000 zooxanthellae per polyp). These anemones were therefore used for short-term monitoring of infection patterns, where densities of residual zooxanthellae were negligible in comparison to densities of phagocytosed zooxanthellae. To keep residual zooxanthellae to a minimum, these anemones were maintained in the dark until inoculation.

**Infection of aposymbiotic Cerrea pedunculatus when coexisting with symbiotic C. pedunculatus**

Under nonstressful conditions, C. pedunculatus regularly releases a small percentage (<0.3% per hour) of its zooxanthellae (Darmayati, 1993). A motile stage has yet to be observed in the life history of these zooxanthellae, either following release or when in culture (Davy et al., in press; Davy, pers. obs.). To establish whether zooxanthellae released by one anemone could infect another anemone, 3-year dark-treated C. pedunculatus (n = 5) were placed in a container with symbiotic C. pedunculatus (n = 5). Three-year dark-treated C. pedunculatus (n = 5) in a second container acted as controls. All anemones were illuminated at 80 μmol photons m⁻² s⁻¹ on a cycle of 12 h light:12 h dark, maintained at 21°C, and fed weekly with Artemia sp. After 8 weeks, the presence or absence of zooxanthellae in the formerly aposymbiotic polyps (which were still distinct due to their lighter pigmentation) was determined. This involved squashing the polyps and using bright-field and epifluorescent microscopy to search their tissues. Densities of zooxanthellae were not quantified.

**Persistence of homologous and heterologous zooxanthellae**

To investigate the extent to which zooxanthellae from different host species could establish a symbiosis with C. pedunculatus, the uptake and persistence of homologous and heterologous zooxanthellae following inoculation were measured.

Three oral discs plus attached tentacles of C. pedunculatus, 5 tentacles from each of 10 individuals of A. pallida and A. viridis, 30 polyps of I. sulcatas, and 10 complete A. pallida were collected for isolation of zooxanthellae. Host tissue for each host species was pooled, homogenized in 10 ml FSW in a glass tissue-grinder, and centrifuged for 10 min at 1200 rpm. The supernatant was discarded, and the algal pellet was resuspended in 5 ml FSW and centrifuged for a further 5 min at 1200 rpm. The supernatant was again discarded and the algal pellet adjusted with FSW to give a concentration of 2 × 10⁵ cells ml⁻¹. Isolates from different host species were kept separate.

Dark-treated specimens of C. pedunculatus (n = 150 and n = 90 for anemones maintained in darkness for 3 years and 5 months, respectively) with oral disc diameters of about 2 to 3 mm were maintained in 100-ml containers (five anemones of the same dark-treatment per container) and inoculated with homologous or heterologous zooxanthellae. Anemones in each container were inoculated with zooxanthellae from just one source, thus preventing cross-infection by zooxanthellae from different sources. A 1-ml hypodermic syringe was used to deposit 0.2 ml of suspension (i.e., 4 × 10⁴ zooxanthellae) onto the oral disc of each aposymbiotic anemone. This number of zooxanthellae was sufficient to saturate the uptake sites of these anemones (Davy, 1994). Once the anemones had expanded again, 0.1 ml of an Artemia sp. suspension was pipetted onto each oral disc. This suspension enhanced ingestion of zooxanthellae (Davy, 1994). To remove any zooxanthellae and Artemia sp. not captured by the anemones, the FSW was then changed. Uninoculated 3-year and 5-month dark-treated anemones (n = 25 and n = 15, respectively; five anemones per 100-ml container) acted as controls for spontaneous infection. All anemones were maintained at 21°C, illuminated with 80 μmol photons m⁻² s⁻¹ on a cycle of 12 h light:12 h dark, and fed weekly with Artemia sp. The densities of zooxanthellae in 5-month dark-treated anemones were measured after 4 h, 2 days, and 4 days, and the densities of zooxanthellae in 3-year dark-treated anemones were measured after 2, 4, 6, 8, and 36 weeks.

Following the incubation period, polyps (n = 4 or 5 for each group; replicate anemones sampled from the same container) were narcotized in 7.5% magnesium chloride.
in FSW. Oral disc diameters were then measured using an ocular micrometer, and oral disc areas were calculated. This method (i) facilitated measurement of large numbers of polyps; (ii) was repeatable; and (iii) gave units (square millimeters of oral disc) proportional to the protein content of the polyp ($r^2 = 0.97$ for anemones with disc diameters of 1.0 to 3.0 mm; Davy, 1994). Polyps examined between 4 hours and 4 days after reinfec-
tion were cut longitudinally, and residual Artemia sp. and zooxanthellae were washed from their enterons; this step was not necessary for anemones examined after 2 weeks or more. Each polyp was then homogenized in 1 ml FSW, and the number of zooxanthellae in the homogenate was counted using a Fuchs Rosenthal hemacytometer. The density of zooxanthellae in the anemone’s tissues was expressed as zooxanthellae per square millimeter of oral disc.

The same method was used to determine the densities of zooxanthellae in five similarly sized, naturally infected C. pedunculatus. These values were compared with those from the infection experiments.

Statistical analysis

Significant differences ($P < 0.05$) were identified using one-way analysis of variance (ANOVA) followed by Bonferroni pair-wise comparisons. When variances were not homogeneous (as determined using Bartlett’s statistic), data were logarithmically transformed.

Results

Infection of aposymbiotic Cereus pedunculatus when co-existing with symbiotic C. pedunculatus

All aposymbiotic Cereus pedunculatus maintained in the presence of symbiotic C. pedunculatus turned brown after 2 months. Light microscopy revealed that this change in color resulted from the presence of zooxanthellae in the endodermal cells. In contrast, control anemones remained white and free of zooxanthellae after 2 months.

Short-term persistence of zooxanthellae

Figure 1 summarizes the patterns of infection of C. pedunculatus by homologous zooxanthellae and by zooxanthellae from Anthopleura hallii, Anemonia viridis, Isozoanthus sulcatus, and Aiptasia pallida over the first 4 days (i.e., over the short-term).

The densities of homologous zooxanthellae were not significantly different from the densities of any of the heterologous zooxanthellae at 4 h (Bonferroni post-hoc ANOVA, $P > 0.05$); but note that the homologous zooxanthellae were 2.5 times denser than the zooxanthellae
from *A. ballii* (Fig. 1A). Subsequently, homologous zooxanthellae persisted at the highest densities. Homologous zooxanthellae were present at significantly greater densities than any of the heterologous strains at 2 days (Bonferroni post-hoc ANOVA, *P* < 0.01).

Short-term infection patterns by heterologous zooxanthellae were similar to one another, with the only major difference being the relatively low uptake of zooxanthellae from *A. ballii*. Zooxanthellae from *A. ballii* were present at significantly lower densities than zooxanthellae from *I. sulcatus* at 4 h (Bonferroni post-hoc ANOVA, *P* < 0.05).

Long-term persistence of zooxanthellae

Figure 2 summarizes the densities of zooxanthellae 2, 4, 6, 8, and 36 weeks after reinfection (i.e., over the long-term), and the density of zooxanthellae in naturally infected *C. pedunculatus*.

Homologous zooxanthellae persisted at significantly greater densities than any of the heterologous zooxanthellae at 2 and 6 weeks (Bonferroni post-hoc ANOVA, *P* < 0.05). Homologous zooxanthellae also persisted at significantly greater densities than zooxanthellae from *A. ballii*, *A. viridis* (Shell Island), and *A. pallida* at 4 weeks, and zooxanthellae from *A. ballii*, *A. viridis* (Shell Island), and *I. sulcatus* at 8 weeks (Bonferroni post-hoc ANOVA, *P* < 0.05). However, at 36 weeks, the densities of homologous zooxanthellae, heterologous zooxanthellae, and zooxanthellae in naturally infected *C. pedunculatus* were not significantly different (ANOVA, *P* > 0.05).

Zooxanthellae from *A. pallida* were present at significantly lower densities than zooxanthellae from *A. viridis* (Lough Hyne) at 4 weeks (Bonferroni post-hoc ANOVA, *P* < 0.05). Zooxanthellae from *A. viridis* (Shell Island) were present at significantly lower densities than any other heterologous zooxanthellae at 2 and 4 weeks, and zooxanthellae from *A. ballii* and *A. viridis* (Lough Hyne) at 6 weeks (Bonferroni post-hoc ANOVA, *P* ≤ 0.02). No other significant differences were evident between the densities of heterologous zooxanthellae (Bonferroni post-hoc ANOVA, *P* > 0.05).

Anemone size

Prior to infection, anemones had oral disc diameters of about 2 to 3 mm. At the end of the 36-week experimental period, all anemones infected with either homologous or heterologous zooxanthellae still had oral disc diameters of 2 to 3 mm and were not significantly differ-
ent in size (average diameter $2.39 \pm 0.03$ mm; ANOVA, $P > 0.95$). As oral disc area is proportional to polyp protein content (see Materials and Methods), these results suggest that changes in anemone biomass were minimal during the experimental period, presumably reflecting the limited feeding regime (once per week). Thus the substantial changes in zooxanthella densities were most likely due to changes in the number of zooxanthellae rather than in the normalizing parameter (oral disc area).

**Discussion**

The results reveal that zooxanthellae from a range of host species, localities, and environments can establish symbioses with *Cereus pedunculatus*. In the short-term, homologous zooxanthellae persist to a greater extent than heterologous zooxanthellae, but over the long-term the two types of zooxanthellae achieve similar densities. The possible reasons for these infection patterns and their implications will be discussed.

**Short-term persistence**

*C. pedunculatus* took up zooxanthellae from *Anthopleura ballii* at a lower rate than zooxanthellae from the other host species, perhaps indicating discrimination based upon the surface characteristics of the zooxanthellae. However, uptake rates of zooxanthellae from the other host species were similar. This suggests that during the initial phases of infection *C. pedunculatus* was unable to discriminate between these zooxanthellae, perhaps because of the similarity of their surface characteristics or the host material contaminating their surfaces (Trench *et al.*, 1981; Colley and Trench, 1983).

Following phagocytosis, all populations of zooxanthellae declined, with the homologous populations declining less rapidly than most of the heterologous populations. Symbiont populations also declined following infections of *Hydra viridissima* (Jolley and Smith, 1980) and the jellyfish *Cassiopeia xamachana* (Colley and Trench, 1983), but did not do so following infections of a number of other species of Cnidaria (Muscatine *et al.*, 1975; Schoenberg and Trench, 1980c; Berner *et al.*, 1993). The decline may represent the nonselective elimination of some types of zooxanthellae more quickly than did heterologous zooxanthellae. Similar patterns have been observed in *C. xamachana* (Colley and Trench, 1983) and *Aiptasia tagetes (= pallida)* (Schoenberg and Trench, 1980c). In contrast, zooxanthellae from *Anemonia viridis* from Shell Island repopulated *C. pedunculatus* more slowly than did any of the other zooxanthellae. These repopulation trends may result from host-algal recognition and greater expulsion or digestion of some types of zooxanthellae than others. However, the repopulation patterns could also be determined by differing growth rates of zooxanthellae; changes in host biomass (either growth or shrinkage), which could cause the ‘dilution’ or ‘concentration’ of zooxanthella populations, were probably not responsible for the density changes. Comparison of growth and expulsion rates of homologous and heterologous zooxanthellae during re-population of aposymbiotic anemones would be an interesting topic for future work.

Several factors could influence the growth of zooxanthellae during repopulation. Firstly, the light and temperature regimes may favor growth by one type of zooxanthellae over another. However, this possibility is not supported by the similarity of the specific growth rates (0.25–0.29 d$^{-1}$) of zooxanthellae from *C. pedunculatus, A. ballii*, and *A. viridis*, when cultured in vitro under the same light and temperature regimes as those used here (Davy, 1994). Secondly, the growth of zooxanthellae may depend upon the ability of a particular strain or species to survive in the intracellular environment of the host (Rahat and Reich, 1988). But this hypothesis is inconsistent with evidence from analogous plant-microbial symbioses (Trench, 1993). Thirdly, hosts may be
able to control the growth of zooxanthellae directly (Smith and Muscatine, 1996). It is therefore possible that *C. pedunculatus* was able to exercise more control over some types of zooxanthellae than others. Finally, the growth of zooxanthellae may be limited by the intracellular space available within the host, with small zooxanthellae achieving greater densities than large zooxanthellae. It seems unlikely that this hypothesis can explain the densities achieved by zooxanthellae from *A. viridis* (Lough Hyne) and *A. pallida* (Fig. 2B), given that these zooxanthellae are similar in size to zooxanthellae from *C. pedunculatus* when in their original hosts, after residing in reinfected *C. pedunculatus* for 36 weeks, and when cultured in vitro (Davy, 1994; Davy et al., 1996, and in press). It is unknown, though, whether these zooxanthellae maintained similar sizes throughout the repopulation process.

In spite of previous differences, at 36 weeks the densities of homologous and heterologous zooxanthellae, and zooxanthellae in naturally infected *C. pedunculatus*, were similar. The decline in the density of homologous zooxanthellae to a “normal” level is unique. If the population of zooxanthellae was unialgal, then the decline may result from the population being “brought under control.” However, why this occurred only several months after the establishment of the symbiosis is unknown. One possibility is that control mechanisms become repressed in aposymbiotic anemones and are restored during the repopulation process. Alternatively, if the homologous population contained a mixture of zooxanthella types, then the decline may result from an initially successful type becoming unstable and being replaced by a type better suited to the culture conditions.

The ultimate success of heterologous zooxanthellae that were initially slow to establish a symbiosis with *C. pedunculatus* can also be interpreted in different ways depending upon the nature of the zooxanthella populations. Firstly, if the populations were unialgal, then host-symbiont adjustment over time may have enhanced the compatibility of the partners (Roughgarden, 1975; Smith, 1980). It would be interesting to re-isolate heterologous zooxanthellae or make reinfected anemones aposymbiotic again, and determine whether the repopulation process is faster when repeated. In a comparable experiment, however, the growth of heterologous zooxanthellae in *A. tagetes* was not enhanced by their previous association with the same host species (Schoenberg and Trench, 1980c). Alternatively, if the populations contained a mixture of zooxanthella types, then the “normal” densities achieved by heterologous zooxanthellae at 36 weeks may represent the proliferation of a small number of zooxanthellae identical to those usually harbored by *C. pedunculatus*. But such a mechanism is unlikely to explain the gradual increase in the density of zooxanthellae from *A. pallida*. This anemone is found at subtropical latitudes in the western Atlantic, and so probably contains zooxanthellae that are quite different from those in *C. pedunculatus*. In fact, the zooxanthellae of this anemone are believed to be a single species, * Syndiodinium bermudense* (Banaszak et al., 1993). Furthermore, evidence from carbon-flux studies suggests that the homologous and heterologous zooxanthella populations were not identical at 36 weeks (Davy, 1994).

**Host-symbiont recombination in the field**

Molecular genetic techniques must be employed to determine the precise nature of the source populations of zooxanthellae and whether heterologous zooxanthellae actually establish symbioses with *C. pedunculatus* in the field. The long-term infection patterns (Fig. 2) suggest that host-symbiont recombination may be possible—if, that is, the new symbiosis is competitive under the prevailing environmental conditions and the heterologous zooxanthellae are not overgrown by homologous zooxanthellae in the short-term (Schoenberg and Trench, 1980c; Colley and Trench, 1983). The ability to establish symbioses with zooxanthellae from a range of sources may enable *C. pedunculatus* to adapt to different environmental regimes, as has been suggested for corals (Buddemeier and Fautin, 1993; Rowan and Knowlton, 1995). Alternatively, it may simply increase the anemone’s chances of survival should it lose all of its zooxanthellae and have to acquire new symbionts. There are, however, no reports of “bleached” *C. pedunculatus* in the field. How zooxanthellae behave in mixed homologous-heterologous inoculations and the ecological advantages of maintaining heterologous zooxanthella populations are important questions for future research, both in temperate and tropical systems.

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