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Effects of the fish anesthetic, clove oil (eugenol), on coral health and growth

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ABSTRACT

Ecological research within coral reefs often requires the use of anesthetics to immobilize organisms. It is therefore important to consider the effect of these chemicals on the surrounding flora and fauna, particularly to the corals themselves. We quantified the effects of clove oil, a commonly used fish anesthetic, on the growth and occurrence of bleaching in three species of corals: *Acropora striata*, *Pocillopora verrucosa*, and *Porites australiensis*. We compared coral responses to five treatments: a gradient of four clove oil concentrations (0-28%) in seawater, and one concentration of clove oil (14%) in ethanol. Each week, we assessed the presence of bleaching, and then applied the treatment. We measured growth over the duration of the 6-week experiment using the buoyant weight technique. Growth and bleaching showed a dose response to clove oil exposure, and the use of ethanol as a solvent had an additional deleterious effect, as also suggested by observed changes in concentrations of eugenol following field application. Overall, growth was reduced by 37.6% at the highest concentration (28% clove oil in seawater) relative to the control (0% clove oil). The reduction in growth was nearly as great (35.3% of the control) at half the concentration of clove oil (14%) when dissolved in ethanol. These results suggest the repeated use of clove oil (even without a solvent) can deleteriously affect corals.

1. Introduction

Coral reefs are among the most diverse ecological systems in the world (Paulay, 1997). To study this diversity biologists often use anesthetics to immobilize fishes and invertebrates for collection, sampling, or experimental removal (Marnane, 2000; Ackerman and Bellwood, 2002; Depczynski and Bellwood, 2004; Wilson, 2005; Valles et al., 2006; Shima et al., 2006, 2008). Field applications of an anesthetic can be particularly useful on rugose or complex reefs where cryptobenthic fish and invertebrates would otherwise remain ensconced (Depczynski and Bellwood, 2004). For some field experiments, anesthetics have been applied repeatedly to the same coral or patch reef to chronically remove fish (e.g., Wilson, 2005; Munday, 2004; Shima et al., 2008). While substantial research has investigated the efficacy and side effects of common anesthetics on fish and humans, there has been relatively little effort to determine their effects on other coral reef organisms, including the corals themselves.

For example, some anesthetics, such as quinaldine, MS-222, 2-phenoxyethanol and benzocaine are limited in their use because of concerns about their health effects on humans or fish (Lewis et al., 1985; Clark, 1990; Brown, 1992). In contrast, clove oil (derived from the plant *Eugenia caryophyllata)* is generally believed to have few health concerns and is used in many countries as a topical anesthetic for humans (Soto and Burhanuddin, 1995). Clove oil varies in its composition, but consists of the phenol eugenol (the active ingredient, making up 70-98% by weight) (Isaacs, 1983) as well as eugenol acetate (>17%), and kariofilen 5 (~12%) (Isaacs, 1983; Soto and Burhanuddin, 1995). Although potentially safe for humans, the environmental effects of clove oil on reef biota have not been well studied (but see Mulochau and Durville, 2004; Frisch et al., 2007).

Many researchers use alcohols or detergents as dissolving agents because clove oil does not easily emulsify with water without vigorous agitation (Munday and Wilson, 1997) (Table 1). Thus, even if clove oil is not harmful, the dissolving agent itself, or the combination of chemicals, may have harmful effects on reef organisms. For example, Mulochau and Durville (2004) found that a clove oil / ethanol mixture reduced the growth and increased the mortality of the branching coral, *Pocillopora verrucosa*. However, these results do not distinguish the effects of clove oil from those of alcohol. More importantly, some field researchers do not use ethanol or detergents due to expense or limited access in remote field locations (Table 1), yet the environmental effects of clove oil emulsified in seawater have never been studied.

Repeated application of anesthetics to the same reefs is often required to maintain experimental manipulations (e.g., Munday, 2004; Wilson, 2005), block experiments through time using the same reefs (e.g., Shima et al., 2008) or describe long-term settlement patterns of small or cryptic species (e.g., Stier and Osenberg, unpublished). Collectors of marine ornamentals might also repeatedly apply anesthetics if they frequent the same collection sites to gather fish for the aquarium trade. Previous studies of the impact of clove oil on corals have addressed single applications using single species of

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Table 1
Summary of field studies using clove oil to study tropical reef fish

Targeted Fish Species	Number (Frequency) of Applications	Concentration of clove oil[a]	Solvent	Reference
multiple tropical fish	7 times (14 days)	20%	ethanol	Depczynski and Bellwood (2004)
multiple tropical fish	3 times (1 year)	23%	ethanol	Ackerman and Bellwood (2002)
Salarias patzneri	1 time	not stated	ethanol	Wilson (2004)
multiple tropical fish	1 time	10%	ethanol	Vigliola and Meekan (2002)
Neopomacentrus filamentosus	14 times (1 day)	10%	ethanol	Vigliola et al. (2007)
multiple tropical fish	9 times (10 days) and 11 times (1 day)	7%	isopropanol/ seawater	Valles et al. (2006)
multiple tropical fish	≥4 times	2%	isopropanol	Wilson (2005) and pers. comm.
multiple tropical fish	1 time	not stated	ethanol	Almany (2004)
Multiple tropical fish	1 time	10%	seawater	Shima et al. (2006) and pers. comm.
Multiple tropical fish	3 times (~12 days)	10%	seawater	Shima et al. (2008) and pers. comm.
Thalassoma quinquevittatum, T. hardwicke	2 times	20%	seawater	Geange and Stier (in review) and pers. comm.
multiple tropical fish	variable	6-20%	ethanol and seawater	Phil Munday, pers. com.

[a] Percent by volume. In some cases, specifics were not provided in the publication, so we contacted the authors for additional detail.

corals (Mulochau and Durville, 2004; Frisch et al., 2007). Thus, there remains a need to 1) assess the effects of repeated exposure of corals to anesthetics, and 2) conduct such a study using multiple species of corals to assess the generality of any observed response.

Here, we compare the response of three species of branching corals to repeated exposure of clove oil (City Chemical LLC, West Haven, CT) consisting of 98% eugenol. Our focal taxa, *Acropora striata, Pocillopora verrucosa,* and *Porites australiensis*, are all common in the lagoons of French Polynesia (Done et al., 1991; Gleason, 1996; Berumen and Pratchett, 2006). We tested four concentrations of clove oil in seawater (0, 7, 14, or 28% clove oil). We also tested one concentration of clove oil in alcohol (14%) to facilitate comparisons with existing studies. We utilized coral fragments (Birkeland, 1976) to reduce variation in responses and thus increase the power of our experimental design.

2. Materials and methods

2.1. Study Site and Experimental Design

Fieldwork was conducted on the northern side of Moorea, French Polynesia (17°30′S, 149°50′W) in Vaipahu lagoon (for a general description, see Galzin and Pointier, 1985). To reduce environmental heterogeneity within the study site, we chose a wide sandy portion of the lagoon with a water depth ranging from 4 to 6 m. We arranged forty-five cinderblocks in three rows of 15 with distances of 3 m between rows and columns (Fig. 1). For each of the three coral species, we collected nubbins (branch fragments) from three different colonies spaced at least 10 m apart to help ensure genotypic variation. Collection sites were chosen for their similarity in depth, light and flow to the study area. Small voucher samples were taken from each colony, bleached, and species identity verified microscopically by examining morphology (Veron, 2000).

On each of three consecutive days, we collected 15 similarly sized nubbins from one colony (genotype) of each species in the early morning, transported nubbins to the lab, mounted them on 7.0×7.5 cm square grids of small (1.5×1.25 cm) plastic mesh vexar using Z-spar™ epoxy (Splash Zone Compound, Kopcoat, Pittsburgh, Pennsylvania, USA), and marked them by inscribing a unique label into the Z-spar™. After the Z-spar™ had cured (approx. 3 h), we determined the buoyant weight of each nubbin (Davies, 1989) and deployed them to the study site in the afternoon. One nubbin from each of the 3 species was attached to each of 15 cinderblocks, grouped by genotype to block for variation in flow, and the relative position of species on cinderblocks was assigned at random (see Fig. 1). We attached a cage made of large (2.5×2.5 cm) square plastic vexar over each cinderblock to prevent predation by corallivores. Other studies conducted using a similar mesh-size demonstrated no difference in growth or survival for nubbins in cages versus cage-controls within a given treatment (White, unpublished). We applied cages to all cinderblocks so any possible effects of cages would not confound our results. This design used 135 nubbins (15 replicates from three

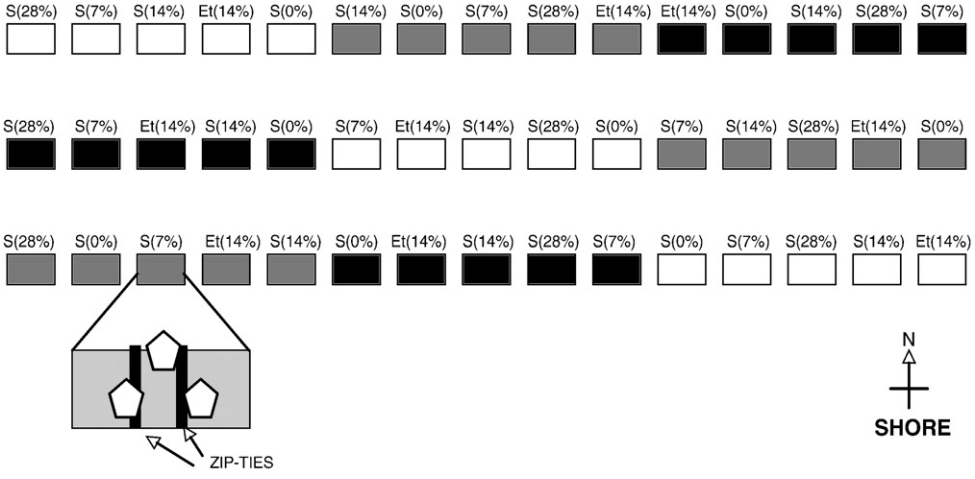

Fig. 1. Schematic of the experimental design: Small squares represent cinderblocks and their numbers correspond to nubbin labels, except where bleached *Acropora* nubbins were replaced. The three shades of grey refer to the genotype blocks. Treatments were randomly assigned within each block. The smaller schematic represents how one nubbin from each species was haphazardly arranged on a given cinderblock.

colonies of each of the three species). After the first day of deployment, five *A. striata* nubbins appeared bleached and were replaced on the third day of outplanting. Nubbins were allowed to acclimate *in situ* for one week prior to the start of the experiment.

2.2. Treatments and Application

The experimental design consisted of five treatments that varied in the concentration of clove oil and the solvent: 7%, 14%, or 28% clove oil in seawater; 14% clove oil in a 90% ethanol solution, and a control of pure seawater (i.e., no clove oil). These concentrations were based on concentrations reported in the literature (Table 1). Squirt bottles were filled with 120 ml of a particular concentration and five stainless steel weights were added to each bottle to facilitate emulsification and mixing. In the field, a bottle was thoroughly agitated and then fully compressed three times while evenly applying the solution across all nubbins on a cinderblock. Treatments were re-administered weekly for five weeks to simulate repeated sampling at a reef, as is done in some fish removal experiments (Table 1). Prior to each treatment application, cages were removed and if any part of a colony showed patchy discoloration, usually associated with the loss of symbionts, it was recorded as bleached. Cages were scrubbed and replaced after each treatment application.

2.3. Collection

One week after the final treatment application, and over three consecutive days, we collected nubbins in the order in which they had been outplanted. We returned nubbins to the lab and thoroughly scrubbed the Z-spar™ bases with a toothbrush to remove fouling organisms. We re-weighed each nubbin and calculated skeletal dry mass (Davies, 1989).

2.4. Duration of Exposure

We outplanted five additional sets (cinderblocks) of nubbins to measure the rate at which clove oil dissipated from the local area. We prepared and dispensed solutions as in the main experiment, except that 1 g of methylene blue was added as well. During application, a second diver collected water samples within 10 cm of the nubbins at intervals of t=5 s, t=15 s and t=25 s (where t=0 s is the onset of treatment application). We did not take data on water flow (e.g. current speed or turbulence), but currents in the lagoon (and our study site in particular) are generally unidirectional. To reduce effects of variation in exposure, we repeated this procedure twice a day for 5 days, randomly alternating the order of treatments applied to each cinderblock using a Latin Square Design through time. We transported water samples to the lab and quantified light absorbance using a Smart™ Spectrophotometer (LaMotte Company, Chestertown, Maryland) at 620 nm wavelength and lagoon seawater from the study site as a blank. We created a calibration curve, based on at least five serial dilutions, to translate the absorbance data into clove oil concentrations (in each case $r^2>0.99$).

2.5. Data Analysis

Growth was estimated as the percent increase of calcium carbonate skeleton accreted for a given nubbin over the course of the experiment (e.g., Miller and Hay, 1998): $100\%\times(\text{Mass}_{\text{FINAL}}-\text{Mass}_{\text{INITIAL}})/\text{Mass}_{\text{INITIAL}}$). Because we did not use bleaching color reference cards (Siebeck et al., 2006), weekly variation in light or turbidity may have limited our ability to document bleaching. Therefore, we averaged across time by calculating the proportion of weeks that a given nubbin was classified as bleached, and then arcsin square root transformed these proportions to normalize the distributions; results were back-transformed for graphical presentation. All analyses were performed using SAS, version 9.1 (SAS Institute Inc., Cary, NC, USA). We compared differences in coral growth and frequency of bleaching using mixed-model ANOVAs. Preliminary analyses indicated no demonstrable effects of genotype or spatial block, so we dropped these terms from the analysis. The resulting model therefore included: species, treatment, cinderblock (random), and the species by treatment interaction. We used Tukey's HSD test to make *a posteriori* comparisons while statistically controlling for multiple comparisons (i.e., maintaining alpha at 0.05). For the analysis of clove oil exposure based on the spectrophotometric analyses, separate one-way ANOVA's were performed for each time period (t=5, 15 and 25 seconds). Variances among treatments were unequal thus we utilized Welch's ANOVA (robust to heterogeneity of variances) followed by *t*-tests assuming unequal variances. We performed these (non-HSD) tests only for the specific comparisons reported.

3. Results

3.1. Coral Growth

Coral growth varied significantly among treatments (ANOVA, $F_{4,78}=9.01$, $P<0.0001$) and among species (ANOVA, $F_{2,78}=36.88$, $P<0.0001$: *A. striata* grew fastest and *P. australiensis* grew slowest); the species-by-treatment interaction was not statistically significant ($F_{8,78}=0.81$, $P=0.60$) suggesting the species were similarly affected by the treatments. Growth averaged across all species was greatest in the control (no clove oil) and declined by 29.7% in response to the application of 14% clove oil in seawater (Tukey's HSD, $P=0.001$), by 35.3% in the 14% clove oil in ethanol treatment (Tukey's HSD, $P<0.001$), and by 37.6% in response to 28% clove oil in seawater (Tukey's HSD, $P<0.001$) (Fig. 2a). Based on post-hoc tests, only the lowest (7%) clove oil in seawater treatment did not differ significantly from the control (Tukey's HSD, $T_{78}=1.97$, $P=0.29$); coral growth at 14% and 28% clove oil (in either seawater or ethanol) did not differ significantly from one another (Tukey's HSD, $T_{78}=0.31$, $P=0.99$), but all were significantly different from the control (Tukey's HSD, $P<0.001$). The highest concentration (28% in seawater) and the 14% in ethanol treatments also differed significantly from the 7% in seawater treatment (Tukey's HSD, $P<0.05$), but the other treatments did not.

3.2. Occurrence of Bleaching

The frequency of bleaching varied significantly among species (ANOVA, $F_{2,80}=11.67$, $P<0.0001$), and among treatments (ANOVA, $F_{4,80}=41.87$, $P<0.0001$), but there was no significant interaction ($F_{8,80}=1.03$, $P=0.42$). In general, the frequency of bleaching increased with increasing concentrations of clove oil and with the use of ethanol as a solvent (Fig. 2b). Bleaching increased from a low of 0-20% of samples in the control treatment to 71-96% of the samples in the 14% clove oil in ethanol treatment. Ethanol had a deleterious effect, as evidenced by the significantly higher bleaching in the 14% ethanol treatment vs. the 14% seawater treatment (Tukey's HSD, $P<0.0001$). The control and 7% clove oil in seawater treatments did not differ significantly from one another (Tukey's HSD, $P=0.99$).

3.3. Duration of Exposure

The 28% clove oil in seawater and the 14% clove oil in ethanol resulted in higher clove oil concentrations at both t=5 s (Welch's ANOVA, $F_{3,16}=9.29$, $P<0.001$) and t=15 s (Welch's ANOVA, $F_{3,16}=7.12$, $P=0.003$) relative to the other treatments, although the concentrations in these two treatments did not differ significantly from one another. The 14% clove oil in ethanol yielded 7.9 times more clove oil after 5 s than the same initial clove oil concentration mixed with seawater (0.111% versus 0.014%, respectively: $t=4.57$, $df=8.55$, $P=0.0002$). After 15 s, the concentration of clove oil in the 14% clove

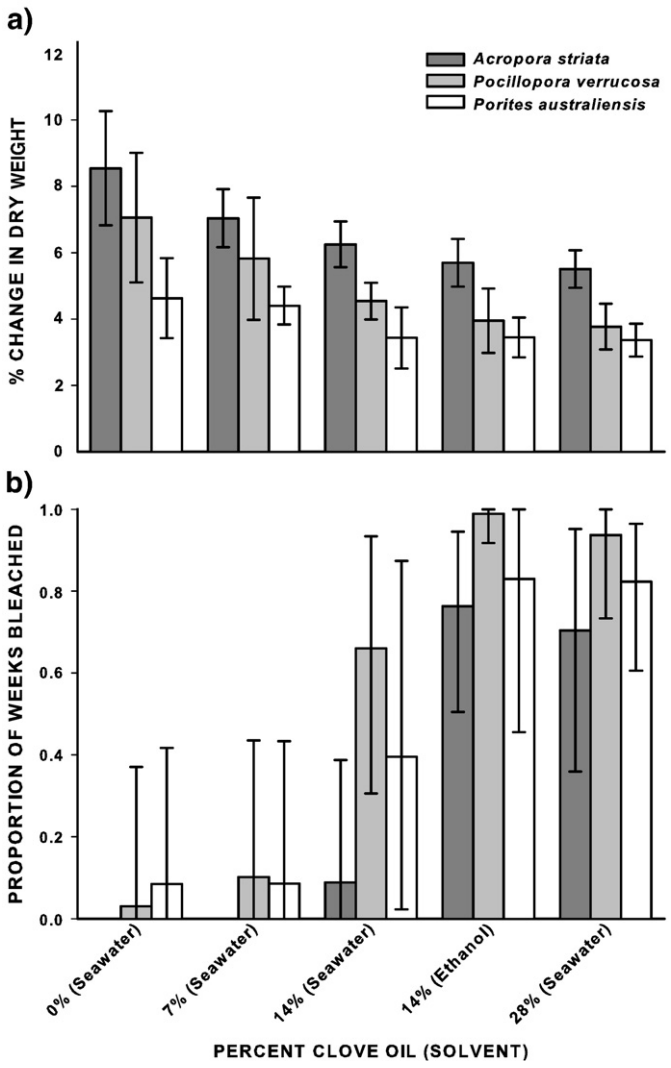

Fig. 2. The effect of different clove oil concentrations (and solvents) on (a) percent change in dry mass (mean ± 95% confidence interval) and (b) proportion of weeks bleached (mean ± 95% confidence interval, back-transformed from arcsin-sqrt transformation). Treatments were applied once a week for five weeks and fragments from each of three species of corals were assessed prior to treatment application the following week. Treatment labels indicate the % concentration of eugenol (0, 7, 14, or 28%) with the solvent given parenthetically (seawater or ethanol).

oil in ethanol treatment remained 11.1 times greater than the concentration in the 14% clove oil in seawater treatment (0.068% versus 0.006%: $t=3.33$, $df=8.19$, $P=0.01$), but did not differ significantly from the 28% clove oil in seawater (0.082%). By 25 s mean clove oil concentration in all treatments was negligible (<0.0008%), except for the treatment with 28% clove oil in seawater (0.007%).

4. Discussion

As new anesthetics are used in field research, their potential effects on the surrounding flora and fauna should be considered (Marking and Meyer, 1985). Our results suggest that repeated applications of concentrations of clove oil ≥ 14% can, on average, reduce growth by 30-40%, and increase bleaching by 20-80%, relative to controls. All three coral species studied were similarly affected by clove oil, suggesting that effects of clove oil may be generic. Although further studies would be necessary to evaluate the mechanism(s) underlying the documented growth and the bleaching responses, our data suggest that these effects are enhanced with the use of ethanol as a solvent. Fortunately, clove oil mixtures of less than 14% in seawater are effective fish anesthetics in the lab and field (Soto and Burhanuddin, 1995; Durville and Collet, 2001; Shima et al., 2006, 2008).

Because our study was based on transplanted coral fragments and not natural colonies of corals, the results may not apply generally to coral reefs. However, previous work indicates that growth rates of experimental transplants of common coral representatives (*Acropora hyacinthus, Pocillopora damicornis,* and *Pavona frodifera*) do not differ from untransplanted controls (Yap et al., 1992). In addition, we allowed transplants to recover and acclimate *in situ* prior to the start of the experiment. Transplants have been shown to experience higher mortality due to predation (Yap et al., 1992); however we eliminated predation by using cages. Finally, because we used small transplants, the nubbins may have been more susceptible to clove oil than larger colonies. We cannot address this concern; further research on the interaction between coral size (or age) and clove oil exposure is warranted.

Our finding of deleterious effects of clove oil-ethanol mixtures on corals under field conditions is consistent with results from other investigators. For example, Frisch et al. (2007) found that colonies of *Pocillopora damicornis* treated with a single application of 100 ml of a clove oil-ethanol mixture (10% clove oil) bleached and incurred tissue mortality. At a lower exposure (a one time application of 10 ml) there was no demonstrable effect. Bleaching and tissue mortality of colonies exposed to ethanol alone (without clove oil) were not significantly increased. Unfortunately, we know of no data on the effect of a single application of clove oil in seawater.

The use of ethanol as a solvent increased bleaching, but did not affect growth, compared to clove oil dissolved in seawater. This result, coupled with Frisch et al.'s (2007) finding that a single treatment of ethanol alone did not affect corals, suggests that ethanol and clove oil may interact, resulting in a mixture more toxic to corals than expected based on the effects of clove oil or ethanol alone. One explanation for the possible synergism may be that ethanol increases the solubility of clove oil in seawater, potentially increasing the permeability of clove oil into the coral tissue. Testing these hypotheses will require a focus on cellular uptake and toxicology, as well as the use of a more complex experimental design in which ethanol and clove oil are varied independently. Our data on duration of exposure for each solution supports the hypothesis of increased solubility and local retention. Ethanol with 14% clove oil and seawater with 28% clove oil yielded the highest (and similar) concentrations of clove oil over time. Furthermore, the concentration of clove oil was approximately 10-fold higher in the ethanol versus seawater treatments (14% clove oil) treatments after 5 s and 15 s.

In our study, using small nubbins in a sandy lagoon, water movement was fairly unidirectional and laminar and water currents quickly dispersed the clove oil downstream of the coral nubbins. In a more structurally complex reef, turbulence and small eddies would likely help retain the clove oil in the reef, thereby increasing exposure of corals to clove oil. Thus, the effects we documented may underestimate those expected under more typical applications. Because clove oil has demonstrable deleterious effects (even in the absence of a solvent), which may be greater than we have documented, we suggest that additional studies are needed that vary the frequency of application, clove oil concentrations, and solvents. Such results could then be used to devise well-informed management scenarios that allow collection with anesthetics but also protect coral reef ecosystems.

Environmental issues aside, these results also point to possible methodological concerns in ecological research. Some field studies measure coral growth as a function of the presence or absence of other organisms (e.g., Myer and Schultz, 1985; Stewart et al., 2006). Our results suggest that the repeated use of clove oil to remove organisms could confound results, if the experiment does not include a proper

control. For example, one could apply clove oil to all reefs, but remove the focal organism(s) from only half of the reefs. In some situations, such a control might not be feasible. In addition, if the effect of clove oil changes the interaction between the focal organism and coral (as might be expected by multiple stressors), then this type of simple controlled experiment would be inadequate and more complicated designs would be required to apply effects estimated in the presence of clove oil to natural situations.

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