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The anaesthetic effect of camphor (*Cinnamomum camphora*), clove (*Syzygium aromaticum*) and mint (*Mentha arvensis*) essential oils on clown anemonefish, *Amphiprion ocellaris* (Cuvier 1830)

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Abstract

The aim of this study was to evaluate the use of clove (*Syzygium aromaticum*), camphor (*Cinnamomum camphora*) and mint (*Mentha arvensis*) essential oils as anaesthetics during the management of clown anemonefish (*Amphiprion ocellaris*). For 15 min, the animals were subjected to concentrations of 5, 10, 20, 27 and 35 μL L^{-1} of clove oil, 17, 35, 50, 70 and 100 μL L^{-1} of mint oil, and 200, 400, 500, 550 and 600 μL L^{-1} of camphor oil (tested in 10 animals per concentration). A control group (without anaesthetic) and a complementary group, which was exposed to ethanol, were also evaluated. After exposure to the anaesthetic, the fish were transferred to clean water to assess recovery. The mortality and feeding behaviour of the fish were then observed for 48 h after exposure to the oils. All of the essential oils produced an anaesthetic effect on *A. ocellaris*. The 27, 70 and 500 μL L^{-1} concentrations of clove, mint, and camphor oils promoted surgical anaesthesia after 310.5, 312.0, and 535.0 s (medians) respectively. The recovery times of fish exposed to these same concentrations were 396, 329.5 and 229 s respectively. The decision of which oil to use is dependent on the management situation and the consideration of the induction and recovery times of each essential oil.

Keywords: eugenol, immersion anaesthesia, menthol, ornamental fish, plant extract

Introduction

Globally, it is estimated that at least one hundred different species of sea fish are produced by breeders for ornamental proposes (Wittenrich 2007). According to the Global Marine Aquarium Database (Green 2008), the most exported marine species between 1997 and 2002 was the clown anemonefish (*Amphiprion ocellaris*). This species alone was responsible for 25% of the total amount of ornamental sea fish trade worldwide (Wabnitz, Taylor, Green & Razak 2003) and is one of the five most imported species by the United States of America (Rhyne, Tlusty, Schofield, Kaufman, Morris & Bruckner 2012). The *A. ocellaris* presents several favourable characteristics that make it exceptionally well suited for rearing in captivity, such as its known ability to reproduce effectively, a high market value and easy adaptation to captivity conditions (Wittenrich 2007; Kodama, Annunciação, Sanches, Gomes & Tsuzuki 2011).

However, activities associated with management during culturing and preparation for trade, such as catching and classifying individuals by size, are stressful (Pedrazzani, Fernandes de Castilho, Carneiro & Molento 2007). These handling actions may result in negative behavioural and physiological effects, such as a decrease in feeding, enhancement of aggressive behaviour and susceptibility to disease (Ross & Ross 2008). The use of anaesthetics during rearing procedures is an alternative to minimize these deleterious effects. Although anaesthesia has been used to immobilize fish, it can also

be utilized to reduce physical damage incurred during handling (Vidal, Furuya, Graciano, Schamber, Santos & Soares 2007) and the associated mortality and morbidity (Cooke, Suski, Ostrand, Tufts & Walh 2004).

Many synthetic anaesthetics have been used on fish, and the most common are MS-222 (tricaine methane sulphonate), quinaldine and benzocaine (methyl-p-aminobenzoate) (Ross & Ross 2008; Neiffer & Stamper 2009). However, these anaesthetics are expensive or may be difficult to acquire (Roubach, Gomes, Fonseca & Val 2005). Because of these complications, the use of vegetal essential oils has emerged as an alternative option for fish anaesthesia (Readman, Owen, Murrell & Knowles 2013). Essential oils are obtained from plants, such as clove (*Syzygium aromaticum*), mint (*Mentha sp.*) and bushy matgrass (*Lippia alba*). These oils have been recommended for fish anaesthesia due to their low costs, easy accessibility, efficacy and environmental safety (Iversen, Finstad, McKinley & Eliassen 2003; Cunha, Silva, Delunardo, Benovit, Gomes, Heinzmann & Baldisserotto 2011).

Several studies have shown the anaesthetic effectiveness of clove oil, which is composed of 70–90% eugenol, when used in freshwater (Inoue, Santos & Moraes 2003; Vidal *et al.* 2007; Oliveira, Carmo, Oliveira & Soares 2009; Simões & Gomes 2009) and marine fish that are intended for human consumption (Souza, Carvalho, Nunes, Scopel, Guarizi & Tsuzuki 2012) as well as in marine (Cunha & Rosa 2006) and freshwater ornamental species (Bittencourt, Souza, Boscolo, Rorato, Feiden & Neu 2012). Similarly, some recent studies have demonstrated the effectiveness of mint oil and its main component, menthol, for freshwater (Gonçalves, Santos, Fernandes & Takahashi 2008; Oliveira *et al.* 2009; Pádua, Pietro, Iglessias-Filho, Ishikawa & Hisano 2010; Teixeira, Moreira, Moreira & Lima 2011; Mello, Costa, Okamura, Ribeiro, Correa & Rosa 2012) and marine fish anaesthesia (Souza *et al.* 2012).

White camphor (*Cinnamomum camphora*) has been used to treat inflammatory diseases, such as rheumatism, bronchitis, asthma, indigestion and muscular pain. It has also been used as a local anaesthetic for humans (Lee, Hyun, Yoon, Kim, Rhee, Kang, Cho & Yoo 2006). Some studies have been performed to examine the immunological effects of stout camphor (*Cinnamomum kanehirae*) essential oil on white shrimp (*Litopenaeus vannamei*) (Yeh, Ya-Li, Shei, Cheng, Huang, Lin & Liu 2009), but there are no studies that have evaluated the anaesthetic effects of these plant compounds on aquatic animals. The purpose of this study was to evaluate the anaesthetic effects of camphor (*C. camphora*), clove (*S. aromaticum*) and mint (*Mentha arvensis*) essential oils and to determine the ideal concentration of each essential oil necessary to safely anaesthetize *A. ocellaris* juveniles during the handling process.

Materials and methods

The experiments were performed in the Research Laboratory of Aquatic Organisms (LAPOA) of the Integrated Group for Studies in Aquaculture (GIA), Federal University of Paraná (UFPR), Curitiba, Parana, Brazil.

Animal acquisition and holding

Two hundred *A. ocellaris* juveniles (0.48 ± 0.21 g, 2.59 ± 0.61 cm; mean ± SD) were acquired from the Azul Fish Farm, São Paulo, Brazil, in February 2013. They were transported in plastic bags that contained water and pure oxygen in a 1:2 ratio. Each bag held 10 fish per litre of water. The bags were shipped in isothermal boxes that were transported by air for 7 h.

In the laboratory, the bags containing the fish were gradually acclimatized over 30 min to avoid differences in water temperature, pH and salinity. Then, the fish were divided and transferred into three glass maintenance tanks (100 cm length × 40 cm width × 50 cm depth) with the back painted black to reduce the effects of incident external light. The fish were fed *ad libitum* twice daily with granulate commercial feed containing 47.5% crude protein (Tetra, Melle, Germany). The remaining food and faeces were syphoned out of the tank 1 h after feeding.

Maintenance of water quality

The tanks were interconnected in a saltwater recirculation system with a protein skimmer and biological filtration as well as a water heater and cooler. The water quality parameters during the experiment were maintained at salinity of 30 ± 0.45 g L^{-1}, temperature 25 ± 0.46°C and pH 7.9 ± 0.4 (mean ± SD). The total ammonia-N was measured every 3 days and never exceeded 0.25 mg L^{-1} or the equivalent of 0.06 mg L^{-1} of

non-ionized ammonia-N ($N\text{-}NH_3$). Water changes were performed weekly by removing 25% of the tank volume and replacing it with clean, properly conditioned water.

Anaesthetic agents

The chemical composition of camphor, clove and mint essential oils were provided by the chemical manufacturer Ferquima Insdústria e Comércio LTDA™ (2013) (Table 1).

Anaesthetic induction

To assess the ideal anaesthetic concentration, a pilot trial was performed for all of the substances at 5 μL L^{-1}. After 15 min of exposure, the fish anaesthesia characteristic behaviour (Table 2) was evaluated. In addition, several concentrations were tested to induce the desired level of anaesthesia (stage IV).

Once the upper and lower limits for each anaesthetic were established, a randomized factorial study was executed for five concentrations of the three essential oils tested. The final anaesthetic concentrations evaluated were 5, 10, 20, 27 and 35 μL L^{-1} of clove, 17, 35, 50, 70 and 100 μL L^{-1} of mint, and 200, 400, 500, 550 and 600 μL L^{-1} of camphor oils. Stock solutions were prepared by dilution at a 1:10 ratio of oil to ethanol (100 g L^{-1} of 100% ethanol). The composition of these stock solutions made it necessary to test the anaesthetic effect of the oil diluent (ethanol) at the maximum concentration used in the dilution processes (35, 100 and 600 μL L^{-1}). The results were compared to a control group in which fish were submitted to the same procedures but not exposed to any anaesthetic.

For anaesthetic induction, 10 fish per concentration were randomly collected from the tanks and transferred separately into glass beakers containing 1 L of saltwater with the established oil concentration, where they were maintained individually for 15 min. During this period, the time required to reach each anaesthetic stage was monitored and recorded (induction period). After the required time and while they were still under anaesthesia, biometric measurements were performed. The weight was obtained using a precision scale (AY 220; Shimadzu, São Paulo, Brazil) and the length was obtained using a pachymeter (Vonder, Curitiba, Brazil).

Five other tanks with 1 L of clean water were used to evaluate the recovery time of the fish exposed to all treatments. The animals that returned to the vertical position and were able to swim were considered recovered. Finally, feeding behaviour and mortality were measured and recorded twice daily for 5 min during the 72 h following oil exposure.

Statistical analysis

Data obtained from each treatment were analysed separately, and the anaesthetic performances of all essential oils were compared. The data normality was evaluated using the Shapiro–Wilk test. The data did not fit into a Gaussian curve; therefore, the anaesthetic induction and recovery times were analysed for significant differences using the Kruskal–Wallis test ($P < 0.05$) followed by multiple comparisons of the mean ranks. Finally, a linear regression analysis was used to evaluate the relation between the biometric parameters of the fish and the observed anaesthetic stages and recovery period (CI 95%). The statistical package used for analysis was Statistica Statsoft™ (V. 10.0).

Results

No anaesthetic effects of fish mortality were exhibited by the control or ethanol-treated groups. Fish exposed to all concentrations of clove, mint and camphor oils reached stages I and II. Stages III and IV were only achieved by the elevated

Table 1 Chemical composition (%) and densities (g cm^{-3}) of camphor, clove and mint essential oils

Essential oil	Main components	Density
Camphor	35.5 1.8-cineole; 30.0 limonene; 13.0 alpha-pinene; 10.0 para-cymene.	0.88
Clove	85.0 eugenol; 13.0 beta caryophyllene; 0.16 alpha copaene + methyl eugenol.	1.04
Mint	37.0 l-menthol; 20.25 menthone; 6.75 limonene; 7.48 isomenthone; 4.60 menthyl acetate; 1.81 isopulegone; 1:39 pulegone; 0.08 carvone; 0.34 cineole.	0.90

Source: Ferquima Indústria e Comércio LTDA.

Table 2 Anaesthetic stages in fish and characteristic behaviour at each stage

Anaesthetic stage	Behaviour parameters
I – sedation	Loss of reaction to touch and visual perception.
II – light anaesthesia	Loss of balance and normal natatory motion interchanged with irregular lateral swimming.
III – deep anaesthesia	Total loss of balance, uncoordinated swimming.
IV – surgical anaesthesia	Reduction in opercular beatment, absence of natatory motion.
V – medullary colapse	Absence of opercular beat, death.

Source: Adapted from Ross and Ross (2008).

concentrations of each product. Only fish exposed to the 35 μL L^{-1} concentration of clove oil registered stage V (medullary collapse; Table 3). At this concentration, four fish died during exposure or within 24 h following exposure.

The induction and recovery times of the concentrations that were induced until anaesthetic stage IV in the majority of fish were compared (Figs 1 and 2). The 20 μL L^{-1} clove oil concentration promoted slower induction to stages I, II and III than did the 35 μL L^{-1} concentration ($n = 10$; $P = 0.035$; 0.040; and 0.013, respectively), even though both provoked surgical anaesthesia (stage IV) in a similar period. The 100 mL L^{-1} concentration of mint oil promoted all anaesthetic stages in *A. ocellaris* at a faster rate than the 50 μL L^{-1} concentration ($n = 10$ per concentration; $P = 0.00$). Stages I, II and IV were also induced more quickly when a higher dosage was used compared to an intermediate dosage (70 μL L^{-1}) (in all stages $P = 0.00$). The three highest camphor oil concentrations (500, 550 and 600 μL L^{-1}) did not present significant differences in the induction time of the anaesthetic stages ($n = 10$ per concentration, $P > 0.05$). In addition, fish agitation was observed during the first moments of exposure to camphor oil. The 27, 70 and 500 μL L^{-1} concentrations of clove, mint and camphor oils promoted surgical anaesthesia in 310.5, 312.0 and 535.0 s (medians) respectively.

Table 3 Number of fish that reached anaesthetic stages under effect of respective concentrations (μL L^{-1}) of clove, mint and camphor essential oils. Also mortality occurred during the 24-h period immediately following anaesthetic induction

Oil	Concentration	Stage I*	II†	III‡	IV§	V¶	Mortality
Clove	5	10	10	0	0	0	0
	10	10	10	10	5	0	0
	20	10	10	10	10	0	0
	27	10	10	10	10	0	0
	35	10	10	10	10	2	6
Mint	17	0	0	0	0	0	0
	35	10	10	0	0	0	0
	50	10	10	10	10	0	0
	70	10	10	10	10	0	0
	100	10	10	10	10	0	4
Camphor	200	10	10	0	0	0	0
	400	10	10	6	3	0	0
	500	10	10	7	5	0	0
	550	10	10	10	7	0	0
	600	10	10	10	9	0	0

*Sedation.
†Light anaesthesia.
‡Deep anaesthesia.
§Surgical anaesthesia.
¶Medullary collapse.

The recovery times of fish exposed to 27, 70 and 500 μL L^{-1} concentrations of clove, mint and camphor oils were 396, 329.5 and 229 s respectively. There was no significant difference among the recorded anaesthetic recovery with regard to all of the clove oil concentrations. However, for mint and camphor oils, an increase in the concentration level produced a more lengthy recovery time. It was also remarkable that the fish exposed to camphor had less variation in the period required to completely recover than those exposed to other products (Fig. 2).

Clown anemonefish subjected to 35 and 600 μL L^{-1} concentrations of clove and camphor oils re-established feeding with 24 and 48 h respectively. There was no observed inhibition in the feeding behaviour of fish exposed to mint oil. The regression analysis indicated that the weight and length did not influence the anaesthetic induction or recovery time in any of the treatments ($P > 0.05$; $r^2 < 0.05$).

Discussion

According to Ross and Ross (2008), anaesthesia in fish should be quickly induced, and the appropriate stage should be achieved in less than

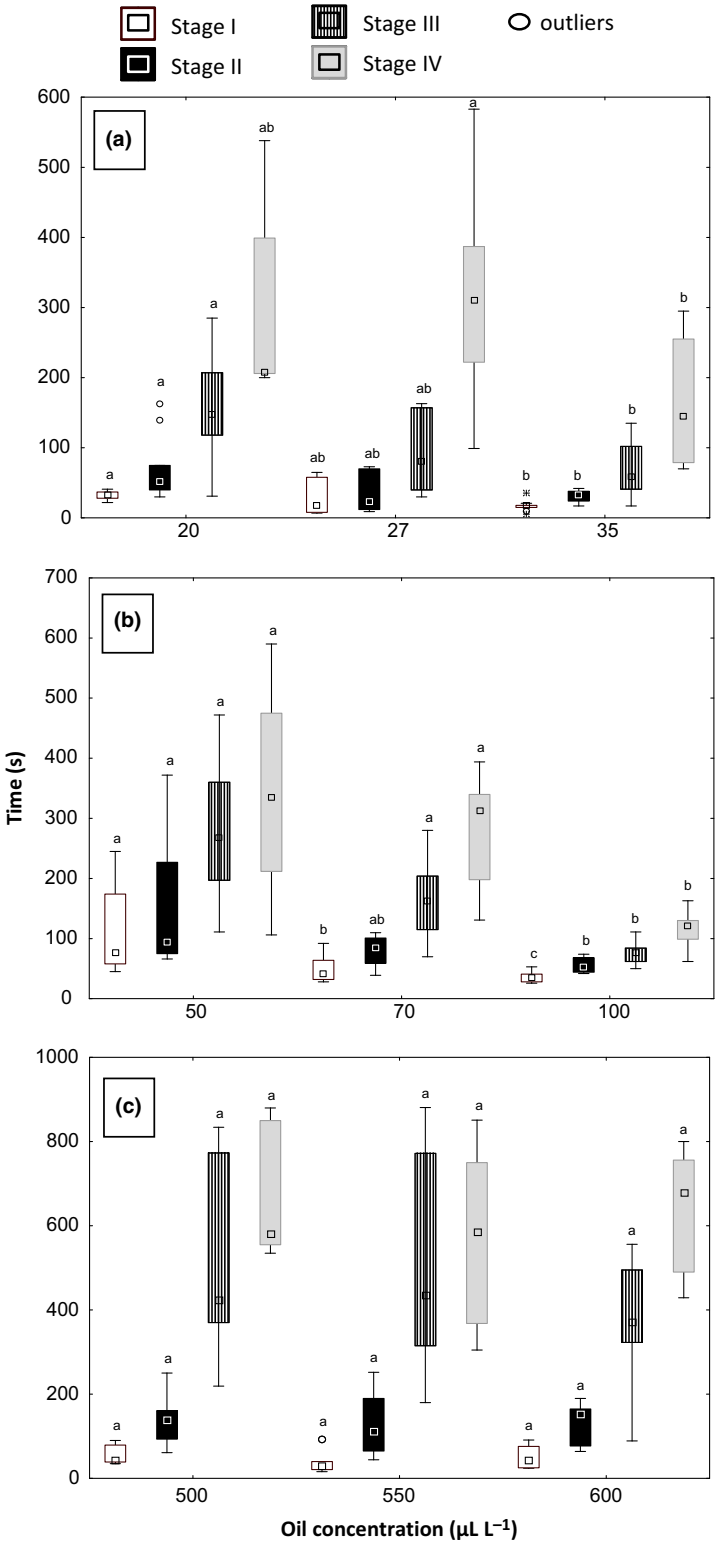

Figure 1 The time required for anaesthetic induction of the first four stages of *A. ocellaris* through the use of clove (a), mint (b) and camphor oils (c). Stage I: Loss of response to external stimuli; II: Partial loss of balance; III: Complete loss of balance; and IV: Reduction in opercular beating. Different letters indicate a significant difference between concentrations ($P < 0.05$).

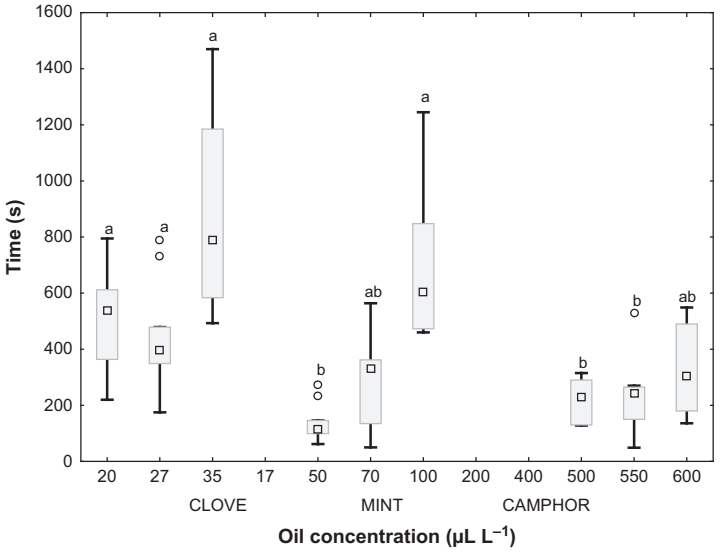

Figure 2 The anaesthetic recovery of *Amphiprion ocellaris* through the use of clove, mint and camphor oils. Different letters indicate significant differences between treatments ($P = 0.00$).

3 min to avoid stress and hyperactive behaviour. The recovery time should also to be no longer than 5 min subsequent to the transfer to clean water. Considering the concentrations that provoked stage IV in most of the animals exposed without causing medullary collapse and extended recovery times, the highest concentrations were 27, 70 and 500 to 550 µL L^{-1} of clove, mint and camphor oils respectively. These doses did not cause mortality. The ideal clove oil concentration established in this study (equivalent to 28 mg L^{-1}) is relatively close to that (50 mg L^{-1}) found for kinguio (*Carassius auratus*) (Bittencourt *et al.* 2012) and pacu (*Piaractus mesopotamicus*) (Gonçalves *et al.* 2008) and is also similar (37.5 mg L^{-1}) to fat snook (*Centropomus parallelus*) (Souza *et al.* 2012). This is also similar to the results found by Cunha and Rosa (2006), who defined 20 mg L^{-1} as the ideal concentration for ornamental sea fish. The same authors did not observe mortality or other adverse behavioural effects in fish after clove oil exposure. In contrast, in this study, some fish died after being exposed to 35 µL L^{-1} (equivalent to 36.43 mg L^{-1}) concentrations of clove oil, which indicates an overdose for clown anemonefish (*A. ocellaris*). In addition, decreased food intake was observed in the remaining fish, indicating stress promoted by the high anaesthetic concentration.

The concentration necessary for anaesthetic induction by mint oil also shows variation between species. Gonçalves *et al.* (2008) showed that 100 mg L^{-1} of menthol is the ideal concentration for pacu (*P. mesopotamicus*). However, for tambaqui (*Colossoma macropomum*) and fat snook (*C. paralellus*), a menthol concentration of 150 mg L^{-1} was suggested by Façanha and Gomes (2005) and Souza *et al.* (2012) respectively. Moreover, for tilapia (*Oreochromis niloticus*), the concentrations that promoted surgical anaesthesia were 120 and 60 mg L^{-1} for juvenile and fingerling respectively (Teixeira *et al.* 2011). This correlates with the 63 mg L^{-1} concentration found to be appropriate for *A. ocellaris* in this study. Interestingly, the recommended mint oil concentration was almost seven times lower than the minimum needed to promote surgical anaesthesia using camphor oil (440 mg L^{-1}). Due to the large volume of camphor oil needed, volatile or water soluble substances present in this oil (easily perceived by their aroma) may have caused an agitation that was registered in the first moments of anaesthetic induction.

Despite the similarity in the optimal concentrations of clove and mint oils necessary for anaesthesia in *A. ocellaris*, it was noted that the times were higher for both anaesthetic induction and recovery than for other species. For example, under optimal concentrations, the induction times for fat snook (*C. paralellus*) and pacu (*P. mesopotamicus*) were 92 and 134 s using clove oil and were 120 and 102 s using mint oil respectively. Cunha and Rosa (2006) suggested that some marine ornamental species are more sensitive to anaesthetics, and frillfin goby *Bathygobius soporator* was the most resistant to clove oil of the seven species tested,

requiring 180 s for induction at concentrations of 20 mg L^{-1}. In contrast, *A. ocellaris* has shown to be even more resistant, needing 310.5 and 312 s to reach stage IV with clove and mint oils respectively. The same tendency of longer times was observed for recovery of *A. ocellaris* and were 396, 329.5 and 229 s using clove, mint and camphor oils, respectively, while other species needed less than 300 s in most situations. This discrepancy may be explained by the differences in water parameters, such as temperature and salinity, or even differences among species (Ghanawi, Monzer & Saoud 2013).

When comparing the oils tested in this study, the rapidity in which clove and mint oils promoted surgical anaesthesia is an advantage related to the use of camphor, especially for use in fast handling cases, such as biometrics procedures. It is necessary to note that while menthol provides a faster induction time, it also increases the risks involved with keeping the fish under anaesthesia for long periods of time. Induction times decreased significantly with an increase in concentration of mint oil.

According to Keene, Noakes, Moccia and Soto (1998), clove oil inhibits the respiratory rate and, consequently, the ability to remove excess anaesthetic from fish, resulting in longer recovery times. Nevertheless, in longer or invasive procedures, such as artificial spawning or surgeries, a longer anaesthesia period would be not only be positive but also necessary (Prince & Powell 2000), with clove oil being the most adequate anaesthetic. Camphor oil may be particularly useful in situations when fish must be exposed to an anaesthetic for longer periods of time but require a fast recovery.

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