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Short Communication

Alternative menthol sources for ascidian relaxation

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Abstract

Proper preservation of ascidians, particularly colonial ascidians, for morphological taxonomy requires specimens to be relaxed before fixation. Menthol crystals, floated in seawater or dissolved in ethanol, are highly effective at relaxing ascidians but can be difficult to obtain on short notice and at remote field sites. We compared the relaxation efficacy and cost per use of three readily available, over-the-counter menthol sources (100% peppermint oil, peppermint extract, and Altoids® mints) to two reagent-grade menthol sources (menthol crystals and menthol dissolved in ethanol). All tested menthol sources successfully relaxed *Ciona intestinalis* individuals to the point that they no longer reacted to a glass probe being inserted in the oral siphon. However, specimens appeared more relaxed when menthol crystals or menthol-in-ethanol were used. Full relaxation is particularly important with colonial species, where contracted specimens may be impossible to identify. Costs per use were generally low for all menthol sources, but menthol crystals can be dried and reused. Thus, peppermint oil, peppermint extract, and Altoids® mints can all be useful substitutes when menthol crystals are unavailable, but menthol crystals remain the best choice when relaxing ascidians for preservation.

Key words: Anesthetization, ascidians, menthol, relaxation, taxonomy

Introduction

When preserving soft-bodied invertebrates for morphological taxonomy, it is vital to first relax or anesthetize the animals so that their muscles will not contract on contact with the preservation solution, obscuring key characters (Williams and Van Syoc 2007). A variety of chemical substances are used for this purpose; certain substances work better for particular groups of organisms (Pollock 1998). For ascidians, a number of chemicals (menthol, MS-222, magnesium sulfate, chloral hydrate, propylene pheoxytol) are effective anesthetics (Pollock 1998). Menthol is most commonly used, either in crystalline form or dissolved in ethanol (Monniot et al. 1991; Lambert 2004; Williams and Van Syoc 2007). It is highly effective at relaxing ascidians, making it an ideal choice for ascidian taxonomists. However, initial collections of unknown ascidians are often made by non-taxonomists, such as more generalized biologists, managers, invasive species monitoring groups, and citizen scientists; these groups generally do not keep a supply of menthol on hand, particularly due to the potentially high cost of pure menthol crystals. Ascidian taxonomists can also find themselves in situations where they are unable to access their laboratory supplies of reagent menthol either because they did not expect to encounter ascidians or they were unable to transport the crystals due to transportation or field site regulations. Here, we tested the ascidian relaxation effectiveness of three sources of menthol that can readily be purchased in stores.

Methods

We compared the anesthetic effect of three over-the-counter menthol preparations on ascidians versus two commonly used reagent-grade menthol preparations. Individuals of the solitary ascidian

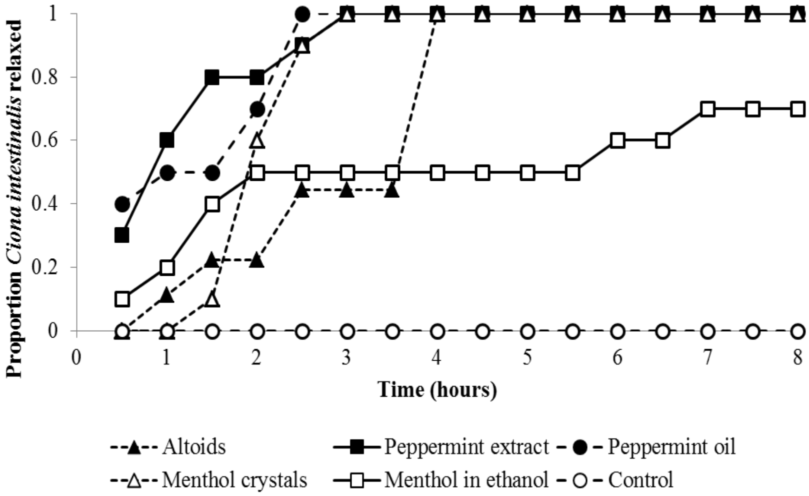

Figure 1. Proportion of individuals relaxed over time after exposure to different sources of menthol. An animal was considered relaxed when there was no visible reaction to inserting a glass probe into the oral siphon. Altoids® (N = 9), menthol crystals (N = 10), peppermint extract (N = 10), and peppermint oil (N = 10) relaxation curves were all significantly different than the control (N = 6) relaxation curve. Menthol-in-ethanol (N = 10).

Ciona intestinalis (Linnaeus, 1767) were collected from holding tanks in the seawater facility at the University of Connecticut – Avery Point in March 2014. In order to reduce artifacts from the difference between ambient water temperature (<4°C) and the room temperature of the laboratory (~25°C), all seawater and *C. intestinalis* used in the experiment were collected and moved to the laboratory at the same time; thus, any changes in water temperature of the course of the experiment would be gradual and the same for all treatments. Animals were individually placed in polypropylene jars containing 400 mL of filtered seawater and allowed to fully extend their siphons before menthol treatments were added. Relaxed length of *C. intestinalis* used ranged from ~3.5 to ~6.5 cm. One of the following treatments was then added to each jar: 1.5 g of 99.7% pure menthol crystals (Acros Organics, Geel, Belgium; N = 10), 0.5 mL of 50% (by volume) pure menthol crystals dissolved in ethanol (Acros Organics, Geel, Belgium; N = 10), 2 mL peppermint extract (< 11% peppermint oil; McCormick and Co., Inc, Hunt Valley, MD; N = 10), 1 mL 100% pure peppermint essential oil (Kis Oils; N = 10), 4 g Altoids® peppermints (Callard and Bowser, Chicago, IL; N = 9), and a menthol-free control (N = 6). Different doses of each menthol source were used to account for different concentrations of menthol in each source. Relaxation was assessed every 30 min for 8 h. An ascidian was considered relaxed when there was no visible reaction to inserting a glass probe into its oral siphon. Between tests for relaxation, jars were tightly covered with screw-on lids. Relaxation curves over time were compared among treatments using a Gehan-Breslow survival curve test (Hosmer et al. 2008) in SigmaPlot v.11, where relaxed individuals were treated as dead and non-relaxed individuals were treated as live. Pairwise comparisons among treatments were conducted using the Holm-Sidak method with an overall significance level of 0.05.

Results

Overall, there were significant differences among relaxation curves (Gehan-Breslow statistic = 26.819, df = 5, $p < 0.001$; Figure 1). Except for the menthol-in-ethanol treatment, all treatments had relaxation curves significantly different than the control (Table 1). The menthol-in-ethanol treatment was the only treatment to have individuals not relaxed after 8 h, though all individuals in this treatment were fully relaxed after 24 h. Among the menthol-added treatments, the only significant difference in relaxation curves was between the peppermint extract and the Altoids® (Table 1), with the peppermint extract relaxing ascidians quicker than the Altoids®. There were no significant differences between the over-the-counter menthol sources and the menthol crystals. Ascidians relaxed the fastest in the peppermint extract treatment (1.25 ± 0.239 h; mean ± S.E.), followed by peppermint oil (1.45 ± 0.293 h), menthol crystals (2.2 ± 0.133 h), Altoids® (3.056 ± 0.403 h), and menthol-in-ethanol (4.2 ± 1.008 h).

Table 1. Pairwise comparisons of relaxation curves among menthol source treatments using the Holm-Sidak method with an overall significance level of 0.05. Greater than (>) indicates relaxation curve of treatment in row was significantly higher (i.e., faster) than the relaxation curve of the treatment in column. Less than (<) indicates relaxation curve was significantly lower (i.e., slower). NS indicates no significant difference.

	Altoids®	Peppermint extract	Peppermint oil	Menthol crystals	Menthol-in-ethanol
Peppermint extract	>				
Peppermint oil	NS	NS			
Menthol crystals	NS	NS	NS		
Menthol-in-ethanol	NS	NS	NS	NS	
Control	<	<	<	<	NS

Table 2. Per-dose cost of menthol sources.

Menthol Source	Dose	Cost per dose
99.7% pure menthol crystals	1.5 g	$0.51[a]
50% (by volume) menthol-in-ethanol	0.5 ml	$0.08[a]
100% peppermint essential oil	1 ml	$0.70[b]
Peppermint extract (< 11% oil)	2 ml	$0.28[c]
Altoids® peppermints	4 g	$0.18[d]

[a]Fisher Scientific 07/01/2014 ($34.34/100 g)
[b]Amazon.com 08/03/2014 ($6.95/10 ml)
[c]Bilo, Statesboro, GA 08/08/2014 ($3.99/29 ml)
[d]Walgreens, Statesboro, GA 08/08/2014 ($2.19/50 g)

Discussion

We found that there are multiple sources of menthol that are effective at relaxing ascidians that are both easily accessible and commercially available. Effective materials included peppermint extract, peppermint essential oil, and Altoids® mints. While all of these sources were effective, there are two potential drawbacks to regular use of these products. The first is cost. Cost per dose ranged from a low of US$0.08 for the traditional, reagent-grade menthol-in-ethanol, to a high of US$0.70 for peppermint oil (Table 2). We note, however, that while menthol crystals were one of the more expensive menthol sources, the crystals do not fully dissolve in seawater, so they can be dried and reused many times. Similarly, recrystallized menthol-in-ethanol can be partially recovered from the surface of the water and re-dissolved in ethanol. In contrast, peppermint oil, peppermint extract, and Altoids® mints cannot be recovered for reuse, potentially resulting in higher cost over time.

The second drawback to over-the-counter menthol products relates to the quality of ascidian relaxation generated by the different menthol sources. All forms of menthol tested were able to relax *C. intestinalis* to the point where the animals no longer visibly reacted when a glass probe was inserted into their oral siphons. However, not all relaxation was equal. Individuals exposed to menthol crystals or menthol-in-ethanol were usually fully extended with completely open siphons. With over-the-counter peppermint oil, peppermint extract, and Altoids®, the animals did not react when poked, but, more often than not, they were still somewhat contracted with partially closed siphons. Partial relaxation is of particular concern with colonial species, where good relaxation is vital for morphological identification.

The relative ineffectiveness of the menthol-in-ethanol treatment was surprising, as this method is commonly used by highly experienced ascidian taxonomists (Lambert 2004). The reduced relaxation level and increased time to relaxation may have been due to an insufficient dose of menthol in the menthol-in-ethanol treatment in our experiments. Only 0.5 mL of the menthol-in-ethanol was used because "about five drops" are reported to be sufficient to induce relaxation (Lambert 2004). However, at 50% menthol by volume, the menthol-in-ethanol treatment had a lower concentration of menthol than the 100% peppermint oil, and the 400 mL of seawater used in this test is likely larger than a "small dish." Additionally, the peppermint oil remained in solution throughout the experiment, while the menthol-in-ethanol quickly re-crystalized (within minutes) and floated on the surface of the water. Re-crystallization could have further reduced the concentration of menthol dissolved in the seawater. Finally, in order to control for timing of menthol addition across treatments, menthol-in-ethanol was added only once, rather than adding more drops at ten-minute intervals, which is suggested by the Lambert (2004) protocol.

In conclusion, menthol crystals remain the gold standard for ascidian relaxation prior to preservation for morphological identification. Menthol crystals are relatively inexpensive, and the same crystals can be dried and reused many times. Ascidians relaxed with menthol crystals are more often fully extended than ascidians relaxed with over-the-counter menthol sources. However, when menthol crystals are not readily available and proper relaxation is critical (such as when investigators unexpectedly locate a suspected new non-native species, a previously unrecorded native species, or a specimen unidentifiable in the field), a number of readily available, over-the-counter menthol alternatives exist. Effective alternatives include peppermint essential oil, peppermint extract for cooking, and Altoid® peppermints. While the particular brands used in this experiment may not be available in all areas, other commercially available products with high concentrations of menthol or peppermint oil (> 11%) can be expected to adequately relax ascidians for preservation for morphological taxonomy.

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References

Hosmer DW, Lemeshow S, May S (2008) Applied Survival Analysis: Regression Modeling of Time-to-Event Data, 2nd Ed. John Wiley and Sons, Inc., Hoboken, New Jersey, 392 pp, http://dx.doi.org/10.1002/9780470258019

Lambert G (2004) Relaxing and fixing ascidians for taxonomy. http://woodshole.er.usgs.gov/project-pages/stellwagen/didemnum/htm/page41.htm (Accessed 20 Jan 2015)

Monniot C, Monniot F, Laboute P (1991) Coral Reef Ascidians of New Caledonia. ORSTOM, Paris, France, 247 pp

Pollock LW (1998) A Practical Guide to the Marine Animals of Northeastern North America. Rutgers University Press, New Brunswick, NJ, 394 pp

Williams GC, Van Syoc R (2007) Methods of Preservation and Anesthetization of Marine Invertebrates. In: Carlton JT (ed), The Light and Smith Manual: Intertidal Invertebrates from Central California to Oregon, 4th Ed. University of California Press, Berkeley and Los Angeles, CA, pp 37–42