

## Comparison of the volatile emission profiles of ground almond and pistachio mummies: Part 2 – Critical changes in emission profiles as a result of increasing the water activity



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### ABSTRACT

Pistachio and almond mummies have been reported to attract adult navel orangeworm moths in field trapping studies. The volatile profiles of these matrices were recently described. Concurrently, recent investigations have demonstrated that the water activity of almond hulls plays an important role in the production of semiochemicals known to attract the navel orangeworm in almond orchards. In the present study, the water activity of pistachio and almond mummies was increased and the resultant headspace volatiles monitored over the course of a week. The volatile profile of wet pistachio mummies contained 86 volatiles, of which 22 were unique to the wet matrix. The volatile profile of the wet pistachio matrix increased in chemical diversity to include small chain alcohols, benzenoids, and fatty acid breakdown products relative to the dry matrix, which primarily emitted terpenoids. The wet almond mummies emitted a total of 57 volatiles, 37 more than dry almond mummies. Among the volatiles detected in the wet almond mummies were three of the five compounds that are found in the synthetic blend of host plant volatiles known to attract navel orangeworm moths. The volatile bouquets from the wet and dry pistachio mummies, and wet almond mummies were evaluated by electroantennographic (EAG) analysis.

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### 1. Introduction

The navel orangeworm (*Amyelois transitella*) has been a major insect pest in California tree nut orchards for the past five decades (Michelbacher and Davis, 1961; Wade, 1961). Egg traps – a plastic cylinder with mesh-covered openings and ridged surfaces that females oviposit upon – have been used to monitor mated female navel orangeworm moth populations in tree nut orchards for ca. four decades (Rice, 1976) and are still considered a standard commercial monitoring tool for females (Burks et al., 2011).

The original bait for egg traps was a modification of the medium used to rear navel orangeworm and included wheat bran, glycerine, honey, and water (Rice, 1976; Rice et al., 1976; Rice

and Sadler, 1977). Rice and Sadler (1977) found that the wheat bran mix had to be kept moist by the addition of water every 3- to 4-days to maintain trap activity. This observation has noteworthy relevance to navel orangeworm attraction in terms of the volatile emission profiles produced by the bait. Recent studies in our laboratories have investigated the complex relationship of fungi and developing fungal spores on almond and pistachio hosts, and the resultant volatiles that appear to be dependent upon the water activity of the host matrix (Beck, 2013; Beck et al., 2011b, 2012a,b, 2014; Mahoney et al., 2014). For example, during the investigation of ground almond hull emissions (Mahoney et al., 2014) the water activity of a dry sample (0.454) was increased to a higher level. Several days after the addition of water the headspace of the sample was again monitored and the volatile profile of the sample had changed substantially in both content and composition.

Nay et al. (2012) recently performed field trapping studies using pistachio and almond mummies – nuts remaining after harvest and aged in the field over the autumn and winter – as bait to attract navel orangeworm moths in pistachio and almond

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orchards. Their results demonstrated navel orangeworm preference for pistachio mummies over almond mummies. More importantly, the results of their study highlighted the need for further exploration of these matrices for potential semiochemicals that could be used in synthetic blends to attract the navel orangeworm. Because of the possible compositional variability of baits such as tree nut mummies (Rice, 1976; Andrews and Barnes, 1982), the volatile composition may be inconsistent and the ensuing field trapping results therefore uncertain (Higbee and Burks, 2011). The compositional variability can include cultivar or phenology differences, larvae infested or non-infested, and frass or fungal bouquets present.

Despite being ubiquitous and generally known, the exact identities and amounts of fungi present on tree nut mummies are not currently documented. The high amount of fungi present on mummies (Fig. 1) and the associated oxidative decay products (Beck et al., 2011b) offer several opportunities for semiochemicals to be emitted and attract navel orangeworm. The volatile emission profiles of the dry matrices of pistachio and almond mummies were reported in Part 1 of this series (Beck et al., 2014), and provided the identities of several potential semiochemicals involved in the chemical ecology of the navel orangeworm. The objectives of this investigation were: (1) to increase the water activity of each of the matrices and determine the corresponding volatile profiles over 1 week; and, (2) to compare the volatile profiles and identify compounds that were shared among the matrices, unique to the wet matrix, or increased in relative amounts as a result of the change in water activity. In addition to analysis of the static headspace, the dynamic headspace volatiles were collected over the course of several days, and the resultant volatile bouquets analyzed by electroantennography (EAG) using antennae from both male and female navel orangeworm.

## 2. Results and discussion

The overarching premise that an increase in water activity of the pistachio and almond mummies would increase the volatile emissions was successfully demonstrated in both matrices. The volatile profiles in each system increased in both composition and content as illustrated in Fig. 2, which shows the wet pistachio mummy volatile emissions increased from 64 total volatiles in the dry matrix to 86 total volatiles in the wet matrix – an increase of 134%. This change in profile resulted in 22 volatiles that were unique to the wet pistachio matrix. The almond mummy volatile emissions increased by 285%, from 20 volatiles in the dry matrix to 57 total volatiles in the wet matrix, and thus 37 volatiles that were unique to the wet matrix. Also, where there were seven volatiles shared between the dry matrices (Part 1, this issue), the wet matrices had 19 volatiles in common including the same seven volatiles detected in the dry matrices, making these seven volatiles



Fig. 1. A typical pistachio mummy (left) and almond mummy (right), both showing fungal growth and decay.

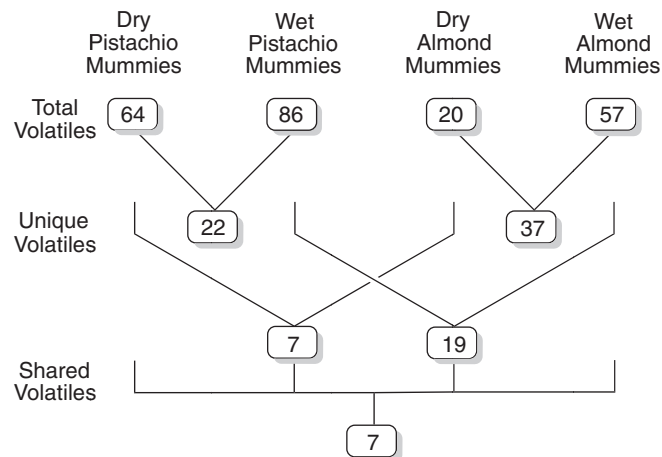


Fig. 2. Illustration depicting the total number of volatiles detected from the four matrices. The row labeled “unique volatiles” shows the number of volatiles detected that were unique to the almond and pistachio matrices when the water activity of each was increased and compared to the respective dry matrices. The row labeled “shared volatiles” shows the volatiles that were common for the dry matrices of almond and pistachio, and those common for the wet matrices of almond and pistachio. The final number at the bottom was the number of volatiles that were common across all four matrices.

( $\alpha$ -pinene,  $\alpha$ -thujene, limonene, 2-pentylfuran,  $\alpha$ -terpinolene, acetic acid, 5,6-dihydro-4-methyl-2H-pyran-2-one) common to all matrices – wet, dry, pistachio, almond (Fig. 2).

The water activity and water content values for the matrices were determined for comparison to the dry mummy matrices. The water activity of the wet pistachio mummies was 0.937 ( $\pm 0.02$  s.e.m.) and the water content 28.28 ( $\pm 0.11$  s.e.m.). The water activity of the wet almond mummies was 0.898 ( $\pm 0.004$  s.e.m.) and the water content 30.41 ( $\pm 0.39$  s.e.m.). The determined water activities of both the wet pistachio and wet almond mummy matrices were well above the needed value of 0.85 for most fungal growth (Hocking, 2001).

The volatiles unique to the wet pistachio mummies, as well as their detected relative abundances over the four sampling periods are provided in Table 1 (see Part 1 for the 64 volatiles from the dry matrix). There were several interesting features of the over-time volatile analyses for wet pistachio mummies. Firstly, volatiles consistently detected throughout all four sampling days (RI = 1090, 1209, 1252, 1387, 1452, 1908) were also in the wet almond matrix, albeit not emitted as consistently in the almond matrix. Secondly, the volatiles with an ethyl ester moiety (RI = 962, 1049, 1065, 1233, 1537, 1639) were all produced on day 3 or 7 of the analysis possibly implying fungal growth, time, or certain concentrations were required to oxidize the corresponding alcohols to acids necessary for esterification. Lastly, presence of methyl ketones and  $\gamma$ -nonalactone (RI = 1077, 1180, 1387, 2021) were similar to the volatiles emitted by almonds during the enzymatic or oxidative breakdown of fatty acids commonly found in both pistachio and almonds (Beck et al., 2011b). Principal component analysis (PCA) of the volatiles from the dry and wet pistachio mummies (Fig. 3) illustrated the differences in emissions over time between the two matrices where significant changes were observed in volatile emission due to the change in moisture. This large separation could be due in part to the volatile emissions from developing fungal spores in the wet matrix and not in the dry matrix (Table 1 and Supplemental Data).

While the number of unique volatiles from the wet almond matrix was greater than the number of unique volatiles from wet pistachio (37 vs. 22, respectively), the sum of all unique wet almond volatile relative abundance ( $1.15 \times 10^8$ ) was essentially equal to the sum of unique wet pistachio volatile relative abundance ( $1.25 \times 10^8$ ). This suggests that on average, the wet

**Table 1**  
Volatiles unique to the wet pistachio mummy profile, relative to the emission profile of the dry pistachio mummies.

Wet pistachio mummy volatiles	RI	Day 0 (3 h)		Day 1		Day 3		Day 7		Sum of rel. abund. $d=0-7$
		Cal'c	Average	(s.e.m.)	Average	(s.e.m.)	Average	(s.e.m.)	Average	
Ethyl isobutyrate <sup>a</sup>	962	0	0	0	0	1,800,085	(303,501)	3,371,757	(650,732)	5,171,842
Ethyl 2-methylbutyrate <sup>a</sup>	1049	0	0	0	0	1,398,760	(205,064)	3,625,331	(849,669)	5,024,090
Ethyl 3-methylbutyrate <sup>a,b</sup>	1065	0	0	0	0	866,667	(133,333)	3,751,780	(407,528)	4,618,446
2-Hexanone <sup>a,b</sup>	1077	0	0	575,208	(59,275)	0	0	0	0	575,208
Isobutyl alcohol <sup>a,b</sup>	1090	539,281	(37,352)	1,267,891	(91,360)	1,866,459	(304,344)	2,872,069	(569,231)	6,545,700
2-Heptanone <sup>a,b</sup>	1180	0	0	836,290	(104,174)	917,881	(165,014)	1,035,458	(286,217)	2,789,629
3-Methyl-1-butanol <sup>a,b</sup>	1209	3,482,087	(386,026)	6,643,989	(378,394)	9,231,808	(877,948)	13,082,904	(2,429,089)	32,440,788
Ethyl hexanoate <sup>a</sup>	1233	0	0	0	0	0	0	460,000	(145,029)	460,000
3-Octanone <sup>a,b</sup>	1252	2,550,000	(132,288)	5,709,667	(127,437)	5,280,000	(271,539)	4,986,667	(254,646)	18,526,333
1-Hexanol <sup>a,b</sup>	1355	0	0	460,351	(47,101)	442,974	(85,968)	326,889	(18,271)	1,230,214
2-Nonanone <sup>a,b</sup>	1387	358,123	(59,387)	1,575,317	(72,063)	1,804,959	(149,248)	2,271,828	(444,397)	6,010,227
3-Octanol <sup>a</sup>	1396	0	0	406,960	(30,519)	285,691	(17,962)	146,667	(31,798)	839,317
1-Octen-3-ol <sup>a,b</sup>	1452	7,062,307	(412,393)	4,987,267	(309,350)	2,500,000	(321,455)	3,466,667	(434,294)	18,016,241
p-Ethylanisole <sup>a</sup>	1524	0	0	210,000	(20,817)	256,667	(71,725)	493,580	(146,453)	960,246
Ethyl nonanoate <sup>a</sup>	1537	0	0	0	0	300,364	(153,110)	631,323	(215,851)	931,686
Isopinocampnone <sup>c</sup>	1541	0	0	807,186	(64,868)	974,152	(74,720)	1,112,373	(131,916)	2,893,710
Dihydrocarvone <sup>c</sup>	1603	0	0	1,302,808	(23,334)	1,512,578	(85,471)	1,848,522	(284,391)	4,663,908
cis-Dihydrocarvone <sup>c</sup>	1622	0	0	524,471	(75,533)	0	0	0	0	524,471
Ethyl decanoate <sup>a</sup>	1639	0	0	0	0	0	0	1,331,031	(41,716)	1,331,031
Ethyl phenylacetate <sup>a</sup>	1782	0	0	0	0	382,374	(43,375)	859,269	(320,536)	1,241,643
2-Phenylethanol <sup>a,b</sup>	1908	655,069	(55,315)	1,763,333	(113,862)	2,655,707	(350,733)	4,338,329	(950,847)	9,412,438
γ-Nonalactone <sup>a,b</sup>	2021	0	0	0	0	0	0	590,135	(39,900)	590,135

<sup>a</sup> Compounds verified by comparison of retention times and fragmentation patterns to authentic standards.

<sup>b</sup> Compounds were also detected in the headspace of wet almond mummies.

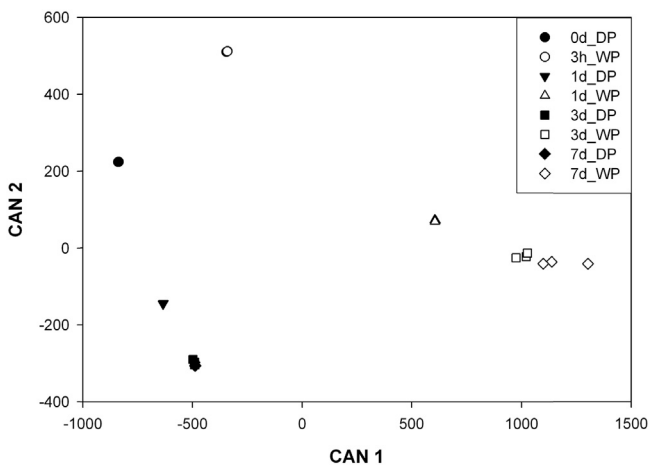
<sup>c</sup> Tentative assignment.

almond mummies were emitting at a lower relative abundance of  $3.1 \times 10^6$  per volatile vs. the wet pistachio mummies were  $5.6 \times 10^6$  per volatile – nearly twice as much per volatile. It should be noted that this does not imply each volatile is produced in this amount or that each contributed equally, but this comparison does exemplify the differences in overall volatile amounts emitted from pistachio and almond matrices. This relatively lower amount of volatile emission was also observed in the study of dry almond mummies (Beck et al., 2014). Table 2 illustrates the intermittent emission of the majority of volatiles from the wet almond mummy matrix. In fact, only six of the 37 volatiles were consistently detected in all four samplings – 1090, 1209, 1280, 1287, 1355, and 1908. Of these six compounds, conophthorin (1287) has been shown to be a critical component of a blend of host plant volatiles that attracts the navel orangeworm in almond orchards (Beck et al., 2012a). Additionally, the production of conophthorin has been shown to be associated with developing fungal spores and that the water activity of the

host is important for its production (Beck et al., 2012b; Beck, 2013; Mahoney et al., 2014). PCA of the dry and wet almond mummy emissions illustrated the substantial changes in volatile profiles between the two matrices (Fig. 4). The similarities in volatile emissions from dry almond mummies were denoted by the tight clustering of all four sampling days, despite some intermittency of volatile production. In contrast, the presence of volatiles unique to the wet matrix and an increase in others over time for the wet almond mummy emissions was demonstrated by the PCA.

In addition to unique compounds generated by the wet matrices, the general increases and decreases in volatile emissions were determined. For brevity, only those compounds that either increased or decreased by a factor of two are listed in Table 3 (see also Supplemental Data). It was notable that a number of compound emissions remained relatively stable despite a change in matrix status. Thirty-nine of the 64 compounds from wet pistachios, seen also in the dry pistachio, only changed by 25% or less. For wet pistachio, a total of eight compounds increased with the majority being oxygenated monoterpenoids, but no relative abundances were produced at more than a five-fold increase. Four compounds decreased in relative abundance. Myrtenal (1623) was the only volatile produced on just 1 day (day 0) and not in the other volatile sampling days. For almonds only two compounds increased in relative abundance, an unknown compound (E, 1666) and benzaldehyde (1516), with the latter increasing 24-fold in the wet matrix. What was interesting about benzaldehyde was that it was produced only on the last day of sampling in the dry matrix and only on the first 2 days of the wet matrix. Large amounts of benzaldehyde are known from almonds (Beck et al., 2011a), yet the large presence under these conditions was noteworthy.

The volatiles common to the wet pistachio and wet almond matrices were determined and are shown in Table 4. The classes/types of compounds of these 19 common volatiles were diverse and included – terpenoids, benzenoids, ketones, esters, alcohols, and lactones. It should be noted that of these 19 volatiles, 12 were also unique in the wet almond, and none are in the dry matrices. Only one compound, 1-octen-3-ol (1452) is a known semiochemical for navel orangeworm (Beck et al., 2012a). In addition to the



**Fig. 3.** Principal component analysis of the first two canonical variables of the volatiles emitted by dry pistachio mummies (DP) and wet pistachio mummies (WP).

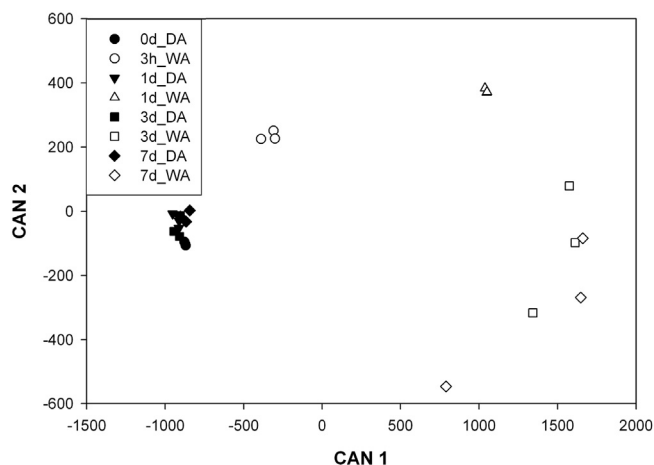
**Table 2**

Volatiles unique to the wet almond mummy profile, relative to the emission profile of the dry almond mummies.

Wet almond mummy volatiles	RI	Day 0 (3 h)		Day 1		Day 3		Day 7		Sum of rel. abund. $d = 0-7$
	Cal'c	Average	(s.e.m.)	Average	(s.e.m.)	Average	(s.e.m.)	Average	(s.e.m.)	
1-Propanol <sup>a</sup>	1036	0	0	372,568	(8,643)	774,384	(145,784)	0	0	1,146,952
Ethyl 3-methylbutyrate <sup>a,b</sup>	1065	0	0	0	0	241,115	(70,568)	0	0	241,115
2-Hexanone <sup>a,b</sup>	1077	0	0	194,681	(70,873)	577,007	(111,471)	564,303	(157,534)	1,335,990
Isobutyl alcohol <sup>a,b</sup>	1090	189,373	(35,245)	1,312,254	(37,893)	2,880,332	(303,001)	2,689,958	(755,407)	7,071,916
2-Pentanol <sup>a</sup>	1120	0	0	0	0	593,468	(166,029)	1,032,942	(341,877)	1,626,409
3-Methylbutyl acetate <sup>a</sup>	1120	0	0	367,230	(21,748)	0	0	0	0	367,230
1-Butanol <sup>a</sup>	1143	0	0	285,764	(80,205)	543,375	(166,767)	0	0	829,139
2-Heptanone <sup>a,b</sup>	1180	0	0	955,642	(97,056)	1,749,110	(249,564)	1,507,164	(251,393)	4,211,916
3-Methyl-1-butanol <sup>a,b</sup>	1209	1,025,360	(134,659)	7,118,106	(96,396)	10,255,266	(1,154,494)	8,409,831	(3,785,102)	26,808,563
2-Hexanol <sup>a</sup>	1222	0	0	0	0	272,573	(9,463)	0	0	272,573
5-Hepten-2-one <sup>c</sup>	1244	0	0	0	0	0	0	167,847	(31,110)	167,847
1-Pentanol <sup>a</sup>	1251	0	0	460,923	(30,583)	355,933	(139,379)	0	0	816,856
3-Octanone <sup>a,b</sup>	1252	0	0	254,843	(28,872)	827,327	(238,763)	1,005,756	(99,782)	2,087,926
Acetoin <sup>a</sup>	1280	418,333	(40,859)	304,667	(55,228)	221,333	(21,987)	363,167	(140,370)	1,307,500
2-Octanone <sup>a</sup>	1283	0	0	906,503	(71,090)	1,185,811	(179,346)	1,028,114	(317,423)	3,120,427
Conophthorin <sup>a</sup>	1287	288,506	(27,109)	669,014	(14,331)	829,121	(37,783)	821,815	(76,944)	2,608,455
2-Heptanol <sup>a</sup>	1322	0	0	240,855	(57,439)	529,350	(68,729)	515,560	(167,906)	1,285,765
Chalcogran #1 <sup>a</sup>	1349	0	0	109,883	(7,515)	102,520	(4,118)	120,545	(22,556)	332,949
Chalcogran #2 <sup>a</sup>	1355	0	0	77,750	(18,276)	143,012	(12,242)	137,989	(13,534)	358,752
1-Hexanol <sup>a,b</sup>	1355	446,010	(16,422)	1,340,630	(112,667)	851,429	(93,419)	648,410	(59,691)	3,286,479
2-Nonanone <sup>a,b</sup>	1387	0	0	320,207	(24,310)	673,203	(34,687)	956,902	(309,345)	1,950,312
1-Octen-3-ol <sup>a,b</sup>	1452	5,001,604	(71,932)	3,178,978	(649,859)	2,372,720	(693,286)	0	0	10,553,302
1-Heptanol <sup>a</sup>	1457	0	0	174,333	(22,995)	0	0	0	0	174,333
Methyl nonanoate <sup>a</sup>	1492	0	0	344,154	(38,192)	0	0	0	0	344,154
2-Ethylhexanol <sup>a</sup>	1492	341,970	(77,341)	0	0	0	0	0	0	341,970
Decanal <sup>a</sup>	1497	404,217	(35,181)	0	0	0	0	0	0	404,217
Methyl benzoate <sup>a</sup>	1617	0	0	892,255	(66,790)	847,532	(292,562)	0	0	1,739,787
Ethyl benzoate <sup>a</sup>	1662	0	0	677,611	(96,730)	896,048	(389,386)	0	0	1,573,658
p-Vinylanisole <sup>c</sup>	1672	0	0	0	0	0	0	3,092,107	(2,020,155)	3,092,107
1,2-Dimethoxy benzene <sup>a</sup>	1723	0	0	0	0	1,065,745	(357,935)	3,404,949	(1,080,862)	4,470,693
2-Phenylethyl acetate <sup>a</sup>	1812	0	0	0	0	328,320	(72,402)	268,821	(59,232)	597,141
2-Acetoxydodecane <sup>c</sup>	1844	0	0	601,885	(68,523)	1,086,910	(194,485)	0	0	1,688,796
Benzyl alcohol <sup>a</sup>	1873	0	0	615,299	(131,638)	0	0	0	0	615,299
2-Phenylethanol <sup>a,b</sup>	1908	1,176,094	(59,032)	5,412,516	(90,036)	7,723,916	(411,520)	6,394,233	(2,169,823)	20,706,759
Methyl eugenol <sup>a</sup>	2010	0	0	223,000	(30,370)	738,000	(248,148)	1,190,000	(238,607)	2,151,000
$\gamma$ -Nonalactone <sup>a,b</sup>	2021	0	0	507,814	(24,538)	0	0	0	0	507,814
3,4-Dimethoxystyrene <sup>c</sup>	2030	0	0	0	0	1,671,930	(648,518)	3,253,897	(456,037)	4,925,827

<sup>a</sup> Compounds verified by comparison of retention times and fragmentation patterns to authentic standards.<sup>b</sup> Compounds were also detected in the headspace of wet almond mummies.<sup>c</sup> Tentative assignment.

diversity of compound types, the relative amounts of the common volatiles varied greatly. When the ratios of the total relative abundances of pistachio to almond were compared they ranged from 0.4 to 409.8 for 2-hexanone/1-hexanol and  $\alpha$ -pinene, respectively.

**Fig. 4.** Principal component analysis of the first two canonical variables of the volatiles emitted by dry almond mummies (DA) and wet almond mummies (WA).

Because the navel orangeworm attractancy to pistachio and almond mummies, presumably both as dry matrices, are known (Nay et al., 2012) we sought to ascertain the electrophysiological response of both male and female navel orangeworm antennae to the volatile bouquets from each of the matrices. Table 5 provides the average electroantennographic (EAG) responses elicited by each matrix. In general, the antennae of male navel orangeworm gave lower numerical EAG response values than those of the female. Both sexes responded with the highest EAG values to the volatile bouquet of the dry pistachio mummies. Unfortunately, the volatile amounts collected for all almond matrices were too low to obtain proper replicates for proper comparisons, despite the use of 50% more material for the dry almond mummies and ground almond kernels. A table of tentative volatiles emitted by almond meal is provided in Supplemental Data. It would be interesting to have determined if the presence of three of the five components from the synthetic blend (3-octen-1-ol, conophthorin, and ethyl benzoate) played a role in the electrophysiological response. Investigations of the volatile bouquets from the varying matrices and bioassays via EAG are being continued. The EAG data are important for two reasons: (1) despite larger sample amounts used for the almond matrices in the headspace Tenax collection the final concentrations of volatiles obtained from the analyses were too low for adequate EAG analysis. This further demonstrates the substantial difference in the amounts of volatiles emitted by the almond matrices relative to the pistachio matrices, and something

**Table 3**

Volatiles from the wet matrices that increased or decreased by more than two-fold relative to their emissions from the corresponding dry matrices.

Pistachio volatiles	RI	% Increase/Decrease	Almond volatiles	RI	% Increase/Decrease
1-Methylpyrrole	1134	485 (+)	2-Ethyl-3-methylbutanal	1064	73 (–)
Thujol	1420	50 (–)	Nonanal	1391	59 (–)
<i>trans</i> -Limonene oxide	1456	226 (+)	Acetic acid	1451	58 (–)
Acetic acid	1451	503 (+)	2-Furfural	1458	57 (–)
Bornyl acetate	1579	53 (–)	Benzaldehyde	1516	2442 (+)
Myrtenal	1623	83 (–)	$\gamma$ -Butyrolactone	1618	66 (–)
<i>trans</i> -Verbenol	1680	61 (–)	Unknown E	1666	218 (+)
1,8-Menthadien-4-ol	1688	205 (+)			
Eucarvone	1695	214 (+)			
Unknown C	1753	228 (+)			
Isopiperitenone	1834	264 (+)			
Piperitenone	1915	425 (+)			

**Table 4**

Volatiles common to both wet pistachio and wet almond mummies. The relative abundances (Rel. abund.) shown are the sums of the averages for all sampling periods. The ratio shown is the relative abundances of wet pistachio (WP) divided by the relative abundances of wet almond (WA).

Volatile identity	RI	Rel. abund. wet pistachio	Rel. abund. wet almond	Ratio (WP/WA)
$\alpha$ -Pinene	1020	649,528,764	1,585,103	409.8
$\alpha$ -Thujene	1024	803,801,009	2,508,981	320.4
Ethyl 3-methylbutyrate	1065	4,618,446	241,115	19.2
2-Hexanone	1077	575,208	1,335,990	0.4
Isobutyl alcohol	1090	6,545,700	7,071,916	0.9
2-Heptanone	1180	2,789,629	4,211,916	0.7
Limonene	1197	2,784,333,320	19,845,743	140.3
3-Methyl-1-butanol	1209	32,440,788	26,808,563	1.2
2-Pentylfuran	1230	4,568,174	298,465	15.3
$\gamma$ -Terpinene	1244	5,531,779	167,847	33.0
3-Octanone	1252	18,526,333	2,087,926	8.9
$\alpha$ -Terpinolene	1281	382,329,245	1,582,333	241.6
1-Hexanol	1355	1,230,214	3,286,479	0.4
2-Nonanone	1387	6,010,227	1,950,312	3.1
Acetic acid	1451	72,682,151	10,427,223	7.0
1-Octen-3-ol	1452	18,016,241	10,553,302	1.7
2-Phenylethanol	1908	9,412,438	20,706,759	0.5
5,6-Dihydro-4-methyl-2H-pyran-2-one	2009	12,776,494	1,150,691	11.1
$\gamma$ -Nonalactone	2021	590,135	507,814	1.2

**Table 5**Male and female navel orangeworm antennal (EAG) responses elicited by volatile bouquet puffs of the treatments<sup>a</sup> shown.

Treatment	Male response ( $\mu$ V)			Female response ( $\mu$ V)		
	<i>n</i> =	EAG	s.e.m.	<i>n</i> =	EAG	s.e.m.
Dry pistachio mummies	6	899.7	92.9	6	1219.9	281.9
Wet pistachio mummies	6	555.0	76.6	6	699.8	156.2
Wet almond mummies	2	243.6	172.2	2	912.2	325.3

<sup>a</sup> Antennal responses to treatments were corrected to the average antennal responses to 50  $\mu$ g of the positive standard acetophenone, 500  $\mu$ V (Beck et al., 2012c).

researchers should keep in mind for trapping experiments or monitoring studies; and, (2) the total amounts of headspace volatiles placed on the assay disc during EAG experiments (50  $\mu$ g) are equal to what is typically used in our laboratory for a single component (Beck et al., 2012c), yet the bouquets assayed comprised multiple volatiles. For example, the wet almond mummy headspace contained 57 volatiles giving on average roughly 1  $\mu$ g of each volatile. This result highlights the importance in finding the components that contributed to the relatively high EAG values provided in Table 5.

The headspace volatile analyses of wet pistachio and almond mummies demonstrated important differences between the two matrices, but also highlighted some key similarities. The results of this investigation provided the identities of several volatiles that may play a critical role in the chemical ecology of the navel orangeworm. The presence of several known navel orangeworm semiochemicals in the wet almond mummy volatile profile

demonstrated the important roles of water activity and fungi in volatile emissions, and also provide a model for elucidation of important semiochemicals from the pistachio matrices. What will be important to consider in future studies is the relative humidity in the orchards and how it affects the water activity of the mummies or tree nuts undergoing mummification during the autumn and winter months while the navel orangeworm is overwintering.

### 3. Experimental

#### 3.1. Almond and pistachio mummy collection

Almond mummies from an orchard containing Nonpareil and Monterey cultivars, and pistachio mummies from an orchard of the cultivar Kerman were collected from the ground and/or trees in the winter months of 2012 and 2013. All mummies were from

commercial orchards located in the southern Central Valley of California (Kern County). The mummies were transported to the USDA-ARS laboratory in Albany, CA in a burlap sack for almonds or brown paper bag for pistachios and kept at room temperature.

### 3.2. Almond and pistachio mummy water and volatile analyses

For each matrix, the pistachio and almond mummies used were from the same sample containers used for the dry mummy study (Part 1, Beck et al., 2014) and identical procedures were used for grinding the samples to powder. To the almond and pistachio powdered materials water, 25% by weight of each sample was added and the mixture stirred for even distribution. The water content of the wet mummy powders was measured via a moisture analyzer (Mettler HB43-S, Columbus, OH) in triplicate 5 g samples, and the water activity was measured via a water activity meter (AquaLab 4TE, Pullman, WA) in triplicate 2 g samples. For collection of volatile headspace 15 g of either almond or pistachio wet mummy powder was transferred to a 4-ounce wide-mouth Mason jar (Ace Hardware, El Cerrito, CA) with a modified lid containing a port for volatile sampling. Triplicates of mummy samples were incubated in a closed system for 7 days at 30 °C. The headspace volatiles of the ground almond or pistachio mummies were adsorbed onto 100 µm solid-phase microextraction (SPME), polydimethylsiloxane fibers (Supelco, Bellefonte, PA). Headspace volatiles of the triplicates were monitored on days 0, 1, 3, and 7 in a closed system, and each at exactly the same intervals of 3 h, 24 h, 72 h, and 168 h. The exposure time of the fiber to the headspace volatiles for each collection was 30 min at 30 °C.

### 3.3. Volatile analysis

All adsorbed volatiles were desorbed and analyzed with identical parameters as noted in a previous publication (Beck et al., 2014). Volatiles were included in the final tables if detected in all three replicates of each sampling period. Volatile identities were verified by comparison of retention time and fragmentation patterns to authentic standards. Volatile identities not verified were listed as tentative assignments and are based on NIST or Wiley databases.

For the dynamic headspace volatile collections, 500 g of ground wet or dry pistachio, or 750 g of ground wet or dry almond matrices were placed in a 12-l flask fixed with a modified lid. A continuous flow of purified air (250 ml/min) was passed through the system and the volatiles collected on Tenax (25 g) for 70 ± 2 h at room temperature. Volatiles were desorbed and concentrated and 1 µl of material injected and analyzed via published methods (Beck et al., 2011a).

### 3.4. Electroantennographic analysis

The experimental procedures (Beck et al., 2012a) and the response normalization process (Beck et al., 2012c) previously described were applied to this study. The solutions of volatiles from the dry and wet pistachio mummy, and wet almond mummy Tenax collections (concentrations of 1.4 mg/ml, 4.3 mg/ml, and 2.4 mg/ml) were used to prepare the bioassay discs at total volumes of 50 µg for EAG puff analysis. The volatile amounts collected from the dry almond mummy and the ground almond kernel experiments (0.05 mg/ml and 0.02 mg/ml, respectively) were inadequate for loading the bioassay discs for EAG analysis.

### 3.5. Statistical analysis

Principal component analysis of each matrix (almond or pistachio) comparing wet and dry samples and time ( $n = 4$ ) was performed using BioNumerics 4.6 (Applied Maths, Inc.). PCA was performed using the log-transformed data of each volatile representing a specific matrix and time point.

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### Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.phyto.2014.01.004>.

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