For: *Biological Control*

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Evaluating Plant Volatiles for Monitoring Natural Enemies in Apple, Pear and Walnut Orchards

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Abstract. The ability to estimate natural enemy abundance is crucial to the integration of biological control into IPM programs. Traditional sampling approaches for natural enemies are few and most are inefficient, but recent studies suggest attraction of natural enemies to plant volatiles may be a useful proxy for direct sampling. We evaluated various combinations of herbivore-induced plant volatiles and floral volatiles as monitoring tools for natural enemies found in apple, pear, and walnut orchards in California, Oregon, and Washington. In 2010 we used a full factorial experimental designs to evaluate lures for all combinations of acetic acid (AA), acetophenone (AP), phenylacetaldehyde (PAA) and 2-phenylethanol (PE). Of nine natural enemy taxa analyzed, we found syrphid flies responded strongly to PE, but combining AA with PE attenuated trap catch and combining PAA to PE eliminated the activity of PE. *Chrysoperla* spp. (Chrysopidae) responded strongly to most of the individual compounds and the various interactions between the components allowed multiple ways to achieve roughly the same trap catch. All of the hymenopteran taxa collected responded strongly to PAA, and PAA containing lures were nearly always a component of the top eight lures. A smaller factorial experiment testing all possible combinations of AA, PAA and methyl salicylate (MS) showed that single component AA or MS lures were generally not significantly different than the controls for all taxa tested, but for the hymenopteran taxa, traps baited with MS+PAA performed the best or were not significantly different than the best performing lure. A 2011 trial was conducted to test the influence of the addition of AA and/or MS on previously tested lures. Combining AA or MS with other lures, improved the capture of *Chrysoperla* spp.; *Scaeva pyrastris* (L.) (Syrphidae) capture was enhanced when MS was used with PE; and PE was attractive to the three syrphid flies, *Chrysoperla* spp., and the parasitoid *Aphelinus mali* (Haldeman) (Aphelinidae). The differential responses to various blends exhibited among taxa show that combinations of plant volatiles can be chosen to increase specificity of attraction to a few taxa or increase the number of species attracted. This flexibility should add to the general value and breadth of use of plant volatile monitoring lures.

Keywords: Herbivore induced plant volatiles; floral volatiles, natural enemy monitoring

1. Introduction

Integrated Pest Management (IPM) depends on a biological control (BC) component to ensure the long-term stability of arthropod management programs (Stern *et al.*, 1959a; Stern *et al.*, 1959b; van den Bosch and Stern, 1962). In most agro-ecosystems, the main BC tactic used is conservation of beneficial natural enemies, where ideally, the timing, intensity, spatial location, and method of pest suppression is carefully designed to have a maximum effect on pest(s) and minimal effects on important natural enemies.

Maintaining conservation BC in an IPM program requires a deep understanding of the phenology of the pests and natural enemies involved and effective ways to estimate their abundance. Only from this information can we determine if BC can suppress a given pest species or if other interventions may be required to prevent economic damage.

Numerous sampling protocols for pest species have been developed for orchard crops (Beers *et al.*, 1994). Many of these sampling protocols are time saving sequential or binomial sampling plans that are efficient and allow IPM specialists to quickly gauge how the pest populations vary over time (Beers and Jones, 2004; Binns and Bostanian, 1990; Binns and Nyrop, 1992; Jones, 1990; Jones, 1994; Nyrop *et al.*, 1989; Nyrop *et al.*, 1999). Attractant traps have also proven exceptionally useful for estimating pest phenology and abundance (Croft and Hoyt, 1983; Riedl, 1980), with huge changes in efficiency occurring since the techniques have improved for the identification and formulation of pheromone lures (particularly for lepidopteran pests). Lure-based attractant trapping has allowed the rapid development of phenology models for pests (Jones *et al.*, 2010; Welch *et al.*, 1978) and the magnitude of trap catch has been used as way to estimate action thresholds for several pests, notably codling moth, *Cydia pomonella* (L.) (Croft and Hoyt, 1983).

IPM practitioners have a much poorer picture of how natural enemy population levels are varying over time and of the diversity of natural enemies present in a system that are suppressing pest populations.

Until recently, there has been little effort to develop trapping protocols to sample beneficial natural enemies and estimate their phenology. However, studies over the last 10 years that have attempted to manipulate natural enemy populations using several herbivore-induced plant volatiles (HIPVs) or floral volatiles (from here on simply called plant volatiles) (James, 2003b; James, 2006; James and Price, 2004; Khan *et al.*, 2008; Simpson *et al.*, 2011) have indirectly show that plant volatiles combined with traps can be used as monitoring tools. HIPV's are generated by a plant in response to herbivore feeding, and multiple natural enemies are thought to use these volatiles to improve host/prey location (de Boer *et al.*, 2008; Dicke *et al.*, 2003; Dicke and Grostal, 2001; Dicke *et al.*, 1990; Fatouros *et al.*, 2005; Vet and Dicke, 1992). While the value of using plant volatiles to manipulate natural enemies is still unclear (James, 2003b; James, 2005b; Jones *et al.*, 2011; Kaplan, 2012; Khan *et al.*, 2008), their use as monitoring tools could potentially lead to better pest management by improving our understanding of natural enemy population abundance, diversity, and phenology (Jones *et al.*, 2009).

Before plant volatiles can be used as reliable monitoring tools, their sensitivity and specificity must be assessed in a variety of situations. The nature of their specificity is particularly important to understand because these kairomones are not species-specific sex pheromones and may be attractive to a wide variety of arthropods. Thus some volatile combinations may catch a diversity of natural enemies that have only minor or no suppressive effects on the pests of interest. Our studies were designed to evaluate several plant volatiles both alone and in various combinations. Our goal was to discover if we could optimize blends for potential indicator species that could be used to evaluate BC services of secondary pests (such as aphids, mites, or scales) in orchards and minimize their undesirable attraction to honeybees. We also evaluated volatile blends for attraction of taxa that occurred in high numbers that may be of interest for ecological or systematics applications beyond the specific orchard systems investigated. Studies were conducted in apple, pear and walnut orchards in the western US.

2. Materials and Methods:

2.1 Lure Construction

Lures were made using 5 cm wide x 7.5 cm long sections of polyethylene tubing (Associated Bag Company, Milwaukee, WI, USA). Tubing was heat sealed at one end to form a bag and a 3.8 cm long piece of dental wick was placed into the bag; the chemical to be tested was applied to the wick and then the open end of the bag was heat-sealed. Only a single chemical was added to the dental wick and we refer to this as a lure. When multiple volatiles were to be tested, multiple lures were placed within a single trap and this is referred to as a multi-component lure. Tubing thickness for lure construction varied by compound; information for each type of lure is given in table 1. All lures were formulated to last at least 40 days at the specified release rate based on unpublished field release rate studies (Jones and Baker, unpublished) and preliminary trapping studies using the methods described by Jones et al. (2011).

2.2 2010 Large Factorial Experiment

A $2 \times 2 \times 2 \times 2$ factorial experiment was set up to test all possible combinations of AA (acetic acid), AP (acetophenone), PAA (phenylacetaldehyde), and PE (2-phenylethanol). These compounds were chosen because of either data already existing in the literature (James, 2003a; James, 2003b; James, 2005a) or from preliminary trapping studies (Jones and Baker, unpublished). The volatiles were set up in a randomized complete block design for three Washington apple orchards (Quincy 1, Quincy 2, Yakima), one California walnut orchard (Woodland), and one Oregon pear orchard (Hood River) with four blocks of traps at each location. Traps within a block were separated by at least 20 m and individual blocks in each orchard were separated by a minimum of 40 m. A control trap, containing a lure to which no volatile components had been added (= -AA, -AP, -PAA, -PE) was included in each block. Traps were deployed for four weeks with positions rotated weekly within a block; traps were always placed in trees adjacent to where traps had previously been hung (either right, left, forward or backward 1-2 trees) to reduce the possibility that the previous lure placement would influence trap catch. Experimental protocols were identical between locations. Timing of trap placement was slightly different for each location because of the different seasonal phenology in each area. Traps were deployed in the field from

31 May to 28 June (California), 28 June to 26 July (Hood River), 15 June to 13 July (both Quincy orchards) and 16 June to 14 July (Yakima).

Lures were hung inside white Suterra LPD (large plastic delta) traps (Suterra LLC., Bend, OR, USA) that are commonly used in orchards for monitoring codling moth. Trap liners were replaced at roughly weekly intervals; when removed, liners were covered with clear Saran™ wrap (S. C. Johnson & Son, Inc. Racine, WI, USA) and frozen at -20°C until specimens could be identified. Taxa identified were primarily natural enemies or honeybees, but a few other insect groups trapped in high numbers were also identified. Honeybees attraction was evaluated because attraction of large numbers of honeybees to a particular lure would potentially reduce the feasibility of using that lure if trap shape, color, or use period could not be adjusted to minimize honeybee mortality. Identifications of natural enemies were made to species for predators and honeybees, and at least to the family level for parasitoids. Lacewings in the genus *Chrysoperla* keyed out as *C. carnea* or *C. plorabunda*, which is considered a species complex by some authors that comprises up to six cryptic species that are differentiated by analysis of their songs (Henry, 1985a; Henry, 1985b; Henry and Wells, 2009; Henry and Wells, 2007; Henry *et al.*, 1993). Because we could not morphologically distinguish the thousands of lacewings captured on sticky traps, we refer to this species complex as *Chrysoperla spp.*

Trap catches for each taxon (species to family) over the four-week trapping period were analyzed using a mixed-effects complete factorial model with normal error distribution and each lure and lure combinations represented fixed effects. Date was coded as 1-4 corresponding to the weekly trap deployment and was considered a random variable (as a repeated measure). Location and blocks within locations and all terms either nested within a random variable (i.e., block [location]) or crossed with a random variable (*e.g.*, any interaction involving location) were considered random (SAS Institute Inc, 2012). Analyses used the restricted maximum likelihood estimation (REML) procedures, which provides a corrected *df*, estimates least square means and provides appropriately scaled standard errors (SAS

Institute Inc, 2012). Raw counts were transformed as $\sqrt{x + 0.5}$ to normalize counts and then transformed counts were divided by the transformed mean for each date for each taxon for each location to generate a normalized trap catch score providing a mean =1. Normalization of the trap catch was used to minimize the possibility that large counts at any particular location might obscure variation at the other locations. Taxa were dropped from analysis for a location if the cumulative trap catch was < 180 individuals over the entire trapping period and weekly catch was dropped if catch was <20 individuals; this ensured that low counts did not distort the analysis. Back-transformed means are presented in all tables and figures.

The factorial structure used allowed us to evaluate the importance of each of the four volatile compounds as main effects (*i.e.*, the statistical factors) and their interactions with one another as well. When interactions were present, they were evaluated to guide the interpretation of the main effects. The lack of significant interaction between a set of factors means that the combined effects of the volatile components can be estimated by just adding their individual effects alone. In contrast, a significant interaction implies either a synergistic or antagonistic effect and suggests caution in the interpretation of the main effects (Quinn and Keough, 2008). The importance of the interaction is nearly always associated with classifying the main effects as not significant rather than the converse, so that it is reasonable to evaluate main effects even when interactions occur (Underwood, 1990; Underwood, 1997). It is important to consider that a particular volatile component may cause either an increase or decrease in trap catch. Because of this, we report the change in trap catch (Δ) between the presence and absence of a volatile component as well the *P* value associated with that comparison, with a positive change indicating that the volatile increased trap catch and a negative change decreased trap catch for a particular taxon. The Δ values reported and shown in the interaction figures are the differences between the back-transformed values.

Significant interactions were plotted to show the magnitude and nature of the interaction (Quinn and Keough, 2008). In addition to interaction plots, the effect of any one volatile component was evaluated

from Δ trap catch in the presence and absence of the other interaction components (Quinn and Keough, 2008). In our study, each lure had only two levels (present or absent), so for example, a significant PAA*PE interaction would have two tests associated with it: (1) Δ trap catch between the presence and absence of PE in the absence of PAA, (2) Δ trap catch between the presence and absence of PE in the presence of PAA. These tests were set up as orthogonal contrasts in JMP (SAS Institute Inc, 2012). Random effects of the model and interactions were tested using the BLUP (Best Linear Unbiased Prediction) and its SEM to construct a *t*-test to evaluate whether a parameter was significantly different from 0. If the parameter was not significantly different than 0, the effect was not considered significant (SAS Institute Inc, 2012).

The protected *lsd* test (only run if a fixed effect was significant at $P \leq 0.05$) was used to evaluate which lures performed the best (Jones, 1984). However, instead of being concerned with separating all possible lure combinations, we focused only on those that were not significantly different from the best lure/lure combination tested for each taxon because anything significantly lower than the best isn't likely to be of interest in the future. However, we also examined the remainder of the *lsd* test to see if there were any trends such as all the best lure combinations having a particular volatile component (or combination of volatile components) present and all the poorest performing lures having some other combination of volatile components.

2.3 2010 Small Factorial Experiment

In addition to the large factorial experiment, we also ran a complete $2 \times 2 \times 2$ factorial set up in a randomized complete block design (four blocks) at a single location in an apple orchard near Quincy, WA from 20 July-10 August. The materials tested were AA, methyl salicylate (MS), and PAA (Table 1). The lure construction, traps used, and analyses (including the data transformation) were the same as discussed above.

2.4 2011 Experiment

The general methods were similar to those reported above with the exception that the traps used were yellow sticky cards (Alpha Scents Inc., West Linn, OR, USA) and that instead of four replicate blocks per location, there were five. The three locations tested were a walnut orchard near Woodland (CA), a pear orchard near Yakima (WA), and an apple orchard near Quincy (WA). Traps were in the field from 26 May–29 June (CA), 20 July–24 August (Yakima), and 12 July–17 August (Quincy).

The 2011 experiments were not factorial, but instead focused on potential new lures and the potential synergistic effects of AA, MS, GER and PE on effective lure combinations that we had tested from earlier studies performed over the 2008–2010 seasons; the only lure component not previously mentioned is geraniol (GER). There were a total of 12 lures evaluated: control (no attractants), AP+GER, AP+PE, GER+MS+PE, AA+ GER+MS+PE, MS, PE, AA+PE, AA+MS+PE, GER+PE, AA+GER+PE, and MS+PE. Because this was not set up as a factorial experiment, a mixed effect ANOVA model was used to analyze the transformed and normalized trap catch data (as above) for each taxon with lure, location, and their interaction as factors. Location, block [location] and date (as a repeated measure nested within block) and the interaction of location and lure were considered random factors, only lure was considered a fixed effect. We also tested the effects of a particular volatile component using contrasts. For example, there were four different contrasts (and an overall contrast) that could be used to test the effect of adding AA to volatile blends (*e.g.*, GER+PE versus AA+GER+PE; PE versus AA+PE; GER+MS+PE versus AA+GER+MS+PE; MS+PE versus AA+MS+PE), five different comparisons for MS, 4 different comparisons for GER, and 2 different comparisons for PE (see table 3 for other specific contrasts). In addition to the individual comparisons for a particular volatile discussed above, there is also an overall test that can be constructed to evaluate the average effect of adding that volatile across all comparisons. These overall contrast tests for the average effect of a volatile are essentially similar in concept to the main effects in a factorial analysis and the individual contrasts can be used to examine (at least roughly) potential interactions similar to simple main effects analysis. To prevent excessive Type I error in the

individual contrasts, we used a Bonferroni correction for the number of comparisons made with a particular volatile component, but the correction was not used for the overall comparison (Jones, 1984). The full ANOVA also allowed us to use a protected *lsd* test for the best lure and the broad trends for importance of the various components as described above.

3. Results

3.1 Large Factorial 2010.

A total of nine different taxa in eight insect families were abundant enough to analyze (Table 2). The apple locations tended to have greater numbers of most taxa than the other crops. However, distinct patterns of taxa were evident among crop type. Walnut orchards had three extremely abundant groups (*Chrysoperla spp.*, parasitoid wasps in the Pteromalidae, and one genus in the Figitidae), but relatively few of the other groups seen in apples and pears. The pear location tended to have much lower levels of most taxa, although large numbers of *Eupeodes fumipennis* (Thomson) (Syrphidae) and moderate numbers of eulophid wasps were captured. Apple orchards had large numbers of parasitoids in the Eulophidae, Figitidae, Pteromalidae, and Scelionidae and moderate numbers of *Chrysoperla spp.*, *E. fumipennis* and *E. volucris* Osten Sacken (Syrphidae). Each taxon is discussed separately below.

Eupeodes volucris and *E. fumipennis* (Diptera: Syrphidae).

The factorial analysis for *E. volucris* showed that the main effects for AA ($\Delta = -0.50$, $F = 8.1$, $df = 1, 4.9$, $P = 0.036$), and PE ($\Delta = 1.43$, $F = 29.8$, $df = 1, 7.96$, $P = 0.0006$) were significant. PE alone produced the highest catch of *E. volucris* and only AP+PE was statistically similar (Fig. 1A). PE was a component of the top six lure combinations, while PAA-containing lures were near or below average. The main effects of AP and PAA were non-significant ($\Delta = 0.01$ and -0.40 , respectively). However, significant interactions were observed for combinations involving PAA and PE. The PAA*PE interaction plot (Fig. 2A) revealed a strong antagonism between PAA and PE where there was a highly significant increase in trap catch in the absence of PAA when PE was added to a trap ($\Delta = 2.87$, $F = 59.5$, $df = 1, 7.8$, $P < 0.0001$). Conversely, in the presence of PAA, trap catch was statistically independent of PE ($\Delta = 0.15$, F

= 0.22, $df = 1$, 7.8, $P = 0.65$). The second significant interaction was AA*PAA*PE (Fig. 2B). In the presence of AA (which inhibits trap catch) the PAA*PE interaction plot drops downward and the response of the flies to the addition of PE in the absence of PAA is attenuated, although the increase was still significant ($\Delta = 2.02$, $F = 28.6$, $df = 1$, 10.9, $P = 0.0002$) compared to when AA was absent (Fig. 2B). When PAA and AA are both present there was no significant difference between the presence and absence of PE in a blend ($\Delta = 0.12$, $F = 0.11$, $df = 1$, 10.9, $P = 0.75$).

Examination of the variance components for the random effects showed that none of the prediction parameters was significantly different from 0, indicating that none of the random effects (location, date, block or the different interactions) were significant.

Eupeodes fumipennis showed a slightly different response to the lure components than did *E. volucris*. The best trap for *E. fumipennis* was with the PE lure alone, and all other lures and lure combinations had a significantly lower trap catch (Fig. 1B). PAA, PAA+PE, AA+PE, and AP+PE traps performed similarly. The remaining lure combinations caught flies at or below the mean trap catch. As before, PE was found in eight of the ten top performing lure combinations. AP or binary combinations of AP (except when mixed with PE) tended to perform below the mean. From the factorial analysis, the main effects of all components except PAA ($\Delta = 0.08$) were significant, although only PE caused a positive change in trap catch ($\Delta = 0.76$, $F = 35.3$, $df = 1$, 10.1, $P = 0.0001$). The AA and AP lures resulted in decreased trap catch (AA $\Delta = -0.55$, $F = 14.7$, $df = 1$, 7.5, $P = 0.006$; AP $\Delta = -0.47$, $F = 11.3$, $df = 1$, 6, $P = 0.015$). However, as would be expected from the reasonable performance of the PAA lure by itself (Fig. 1B), there were several significant interactions that modify the interpretation of the results. The AA*AP interaction was significant ($F = 5.3$, $df = 1$, 968, $P = 0.021$) and is characterized by both materials causing a reduction in trap catch (Fig. 2C). The interaction occurs when AP is present in combination with AA – in this case the reduction in trap catch caused by AA is attenuated by the reduction associated with AP ($\Delta = -0.25$, $F = 2.7$, $df = 1$, 11.3, $P = 0.12$). But when either component is added in the absence of the other, there are

significant declines in trap catch (AP alone $\Delta = -0.71$, $F = 16.6$, $df = 1, 11.3$, $P = 0.002$; AA alone $\Delta = -0.79$, $F = 20.1$, $df = 1, 13.7$, $P = 0.0005$). The AP*PE interaction was also highly significant ($F = 10.8$, $df = 1, 968$, $P = 0.001$) (Fig. 2D). PE in the absence of AP resulted in a significant increase in trap catch ($\Delta = 1.11$, $F = 44.8$, $df = 1, 20.8$, $P < 0.0001$), but the increase was reduced when AP was present ($\Delta = 0.45$, $F = 9.8$, $df = 1, 20.8$, $P = 0.005$). Similar to that observed with *E. volucris*, the PAA*PE interaction was very strong and showed the classic crossed pattern of a severe interaction (Fig. 2E). The inhibition of the activity of PE in the presence of PAA was virtually complete ($\Delta = 0.08$, $F = 0.24$, $df = 1, 20.8$, $P = 0.63$), compared to a large increase in the absence of PAA ($\Delta = 1.43$, $F = 88.7$, $df = 1, 20.8$, $P < 0.0001$). The AP*PAA*PE interaction (Fig. 2F) was similar to the PAA*PE interaction in the absence of AP, but in the presence of AP, the interaction plot showed the increase associated with PE in the absence of PAA was diminished dramatically. Essentially, the AP masked the activity of PE in a way similar to PAA, and makes the interaction plots significantly different with and without AP (Fig 2F).

Examination of the variance components of the random effects showed any interactions involving locations were $< 2.4\%$ of the residual variance after the variance due to fixed effects was removed. None of the predicted parameters were significantly different from 0, indicating that none of the random effects (location, date, block or the different interactions) were significant.

Chrysoperla spp. (Neuroptera: Chrysopidae). The traps with the highest catch of *Chrysoperla* spp. always had multi-component lures, with all single component traps performing below average (Fig. 1C). AA was present in the top five lure combinations, even though it was not significantly different than the unbaited control as a single component trap. The factorial analysis revealed that the main effects of all components were all highly significant and caused increase trap catch of roughly the same magnitude (AA $\Delta = 0.91$, $F = 22.7$, $df = 1, 5.0$; AP $\Delta = 0.78$, $F = 43.5$, $df = 1, 13.9$; PAA $\Delta = 0.79$, $F = 23.3$, $df = 1, 4.5$; PE $\Delta = 0.80$, $F = 48.5$, $df = 1, 3.8$). However, there were four significant two-way interactions and one three-way interaction to consider. The AP*PAA interaction ($F = 5.9$, $df = 1, 13.9$, $P = 0.03$; Fig 3A)

showed a non-additive interaction, both components on their own improving trap catch over the control by roughly the same rate ($\Delta \approx 0.98$). Addition of either component to the other resulted in an additional increase in trap catch at roughly half the rate of the single components by themselves (i.e., $\Delta \approx 0.54$). The AA*PE interaction ($F = 13.6$, $df = 1$, 20.5 , $P = 0.001$; Fig. 3B) showed strong synergism, with both components increasing trap catch by themselves in a similar magnitude ($\Delta = 0.54$ and 0.43 , respectively) compared to the base level (i.e., no AA or PE). However, when AA and PE were together, trap catches increased nearly 1.8 fold ($\Delta = 1.77$) greater than what would be expected if two components acted additively (i.e., $\Delta = 0.54 + 0.43$). AA did not have a significant interaction with either AP or PAA, but acted additively with them. The AP*PE interaction (Fig. 3C) was similar to the AP*PAA interaction in that both increased trap catch the same amount ($\Delta \approx 1.15$) by themselves and there was a slight ($\Delta \approx 0.33$) increase in trap catch when both attractants were present in a blend compared to either component by itself (Fig. 3C). The PAA*PE interaction (Fig. 3D) likewise follows the same pattern, with both components being attractive by themselves ($\Delta \approx 1.55$), but when both components were present there was no significant difference in trap catch ($\Delta \approx -0.16$). The significant AA*PAA*PE interaction is the result of the presence of AA shifting the entire PAA*PE interaction upwards, and the synergistic effect of PE and AA in the absence of PAA (Fig. 3E).

Because all the main effect tests showed significant increases related to each component, it is clear that optimal trap catch of *Chrysoperla spp.* requires a multicomponent lure, but the interactions of the different components allow multiple ways to achieve optimal trap catch. From the factorial analysis, it appears that PAA, PE, and AP all potentially share a common mode of action and would be relatively interchangeable in a multicomponent lure, with some benefits with the PE+AP combinations over those with PE+PAA. While AA acts additively with AP and PAA, it acts synergistically with PE, so blends with PE+AA should be relatively efficacious and the data show that four of the top five lures had this combination (Fig. 1C). The AA+AP+ PAA trap was the most attractive, although the *lsd* test showed that it was not significantly different from the AA+AP+PE or AA+AP+PAA+PE lure combinations. From the

standpoint of using the fewest components, either the AA+AP +PAA or the AA+AP +PE combinations would give optimal attraction without the need of a fourth component. None of the two-component lures were as good as the top two three-component lures, but the AA+PE lure would again be a good second choice considering the number of components required.

As with the two syrphids, evaluation of the variance components showed none of the model parameters were significantly different from 0, indicating that the random effects (location, date, block or their interactions) were not significant.

Parasitic Hymenoptera. A total of five different taxa of parasitic Hymenoptera were abundant enough to analyze. Four of the different taxa (Eulophidae, Figitidae, Scelionidae, and Pteromalidae; Fig. 4A-D) responded very strongly to the PAA component. For all four taxa, the top eight lures always contained PAA, and only in the case of the eulophids and figitids was PAA by itself significantly different than the best performing lure. In addition to their response to PAA, figitids also responded to the presence of AP, which was found in the top four lure combinations.

The factorial analyses of the eulophids, figitids, scelionids, and pteromalids were all very similar. The main effects of PAA were all highly significant and caused large increases in trap catch ($\Delta = 5.93, 5.32, 3.93, \text{ and } 4.91$, respectively). The main effect of AA was never significant, but AP showed significance for the eulophids ($\Delta = 0.53, F = 6.3, df = 1, 6, P = 0.046$) and figitids ($\Delta = 2.62, F = 13.9, df = 1, 5.7, P = 0.01$). The main effects of PE never reached statistical significance and only the PAA*PE interaction was significant, and only for the eulophids (Fig. 5A) and scelionids (Fig. 5B). Examination of the interaction plots for both families shows the plots are nearly identical, and the effect of PE alone was minimal (although significant) (eulophids $\Delta = 0.46, F = 8.07, df = 1, 6.75, P = 0.026$; scelionids $\Delta = 0.47, F = 9.15, df = 1, 473.9, P = 0.003$) and non-additive in the presence of PAA. For all four families, evaluation of the random effects variance components did not show significant location or date interactions.

The ichneumonids were the fifth hymenopteran taxa that were caught in significant numbers, but only at the Yakima location. This group responded to lures slightly differently than the other four families. In contrast to the other taxa, the top seven lures were multicomponent, with eight of the top nine lures containing PE and the top three also containing PAA, and the top two also contained AP (Fig. 4E). The factorial analysis follows from the lure ranking where the main effects of AP, PAA, and PE were all significant ($\Delta = 0.56, 0.68, \text{ and } 1.24$, respectively), but AA was not. There were no significant interactions, nor were there any significant random effects.

Honeybees. Trap catch in lures containing PAA was enormous ($>3,400$ bees caught at 4 of the 5 sites and 17,800 caught at the highest site over a four week period well after bloom – Table 2) - although at least part of this was a function of using the white delta traps which are attractive to honeybees (Knight and Miliczky, 2003). Similar to the eulophids, figitids, scelionids, and pteromalids, PAA was a component of the top eight lure combinations, and no trap was significantly better than PAA by itself. PE was also a component in the top four lure combinations (Fig. 4F). The single-component AA, AP and PE lures caught well below the mean at all locations. The factorial analysis showed that the main effects of PAA and PE significantly increased trap catch ($\Delta = 27.45$ and 6.52 , respectively), but AA and AP did not ($\Delta = -0.34$ and 1.84 , respectively). This picture is modified by the two significant interactions, both of which involved AP*PAA. The two-way interaction (Fig. 5C) showed AP caused a significant increase in trap catch in the absence of PAA ($\Delta = 3.61$), but there was a non-significant decrease ($\Delta = -2.91$) in the presence of PAA. The significant AP*PAA*PE interaction (Fig. 5D) mirrored the AP*PE interaction in the absence of PE, but in the presence of PE the interaction shifted upwards. For both the two and three-way interactions, it is obvious that PAA dominated the interaction plot; in the absence of PAA, any of the components increased trap catch compared to the base level (no PAA, AP or PE) and the effects appeared additive. However, in the presence of PAA, none of the components increased trap catch significantly

over what was found in single lure PAA traps, which shows the components do not act additively.

Evaluation of the random effects showed no significant location or date effects.

3.2 Small Factorial 2010

Eupeodes fumipennis. The best lure for *E. fumipennis* was PAA, but no lure caught significantly more flies than the unbaited control (Fig. 6A). Any lure containing AA, other than the three-component lure, caught fewer flies than the overall mean. As in the large factorial test, the main effects of AA were statistically significant ($\Delta = -1.36$, $F = 6.9$, $df = 1$, 77, $P = 0.01$). The main effects of PAA ($\Delta = 0.18$, $F = 0.13$, $df = 1$, 77, $P = 0.73$) and MS ($\Delta = 0.44$, $F = 0.74$, $df = 1$, 77, $P = 0.39$) were not significant, but the three-way interaction (AA*MS*PAA) was significant ($F = 5.3$, $df = 1$, 77, $P = 0.02$). Examination of the simple main effects revealed that the significant interaction was the result of AA suppressing the MS alone treatments ($\Delta = -2.19$, $F = 4.0$, $df = 1$, 77, $P = 0.049$) or PAA alone ($\Delta = -3.10$, $F = 8.7$, $df = 1$, 77, $P = 0.004$).

Parasitic Hymenoptera. The families Eulophidae, Figitidae, Pteromalidae, and Scelionidae were abundant enough to analyze in the parasitic Hymenoptera. The best combinations of lures for all four families contained PAA, and MS+PAA was either the best trap or not significantly different from the best lure combination. Although there is some variability, all PAA containing traps were generally better than those not containing PAA (Fig. 6B-E). The factorial analysis showed that the main effect of PAA was highly significant ($P < 0.0001$) for the families Eulophidae ($\Delta = 10.43$, $F = 294.3$, $df = 1$, 77), Figitidae ($\Delta = 2.08$, $F = 68.7$, $df = 1$, 77), Pteromalidae ($\Delta = 8.88$, $F = 300.9$, $df = 1$, 77), and Scelionidae ($\Delta = 37.08$, $F = 641.6$, $df = 1$, 77). For the eulophids and scelionids, the main effect of MS was also significant ($\Delta = 2.20$, $F = 13.1$, $df = 1$, 77, $P = 0.0005$ and $\Delta = 5.89$, $F = 16.2$, $df = 1$, 77, $P = 0.0001$, respectively), but for the other families, MS effects were not significant. The main effect of AA was not significant for all families and there were no significant interactions in all four families.

Honeybees. For the honeybees, any traps containing PAA were statistically equivalent to each other and all PAA containing traps were significantly better than those not containing PAA (Fig. 6F). Only the

PAA main effect was significant for honeybees ($\Delta = 17.39$, $F = 277.9$, $df = 1, 77$, $P < 0.0001$). None of the other main effects were significant, nor were there any significant interactions.

3.3 2011 Experiment

Eleven taxa occurred in high enough numbers to allow statistical analysis but only seven showed significant lure effects (Table 3). Those taxa not showing significant lure effects were the ceraphronids, *Trechmites insidiosus* (Encyrtidae), the ichneumonids (lumped together), and the scelionids. The lack of response to the volatiles probably reflects not only the choice of volatiles used, but also the fact that this test used yellow panels instead of the white large plastic delta traps. Thus, some of the effect of the volatiles may have been swamped out by the visual attraction of the yellow panel.

Eupeodes volucris. The *lsd* test showed that the top nine lures for *E. volucris* all contained PE, with the top five lures not showing significant differences (Fig. 7A). The contrasts showed no significant changes were associated with AA, MS, or GER, but the PE contrasts showed significant increases in trap catch associated with addition of PE to a lure (Table 3). The importance of PE as an attractant agrees with the results from the 2010 large factorial study, although the lack of significance of the AA component contradicts its significant negative impact in the 2010 factorial study.

Eupeodes fumipennis. The *lsd* test showed that the best trap for *E. fumipennis* was with AP+GER, although there were no significant differences between that lure combination and the next three lure configurations (Fig. 7B). The top three traps all had GER in common, and seven of the top eight had PE present. The trap catch of any trap containing AA caught fewer flies than the mean.

The contrasts showed that AA caused significant reductions in trap catch compared to the same trap configurations without the AA lure present. Only the PE versus PE+AA comparison was not significant (Table 3). Addition of MS did not result in a significant change in trap catch for any of the comparisons or for the overall comparison of MS effects. GER had a significant positive effect on trap catch overall,

although the only single comparison that was significant was the PE versus PE+GER comparison (Table 3). Overall, PE also caused a significant increase in trap catch, although the control versus PE comparison was not significant. This is different from the 2010 large factorial where PE was the best lure for *E. fumipennis* and is likely related to the use of the yellow cards that have a strong visual component compared to the white large plastic delta traps used in 2010.

Scaeva pyrastris (L.) (Syrphidae). The best performing trap for *S. pyrastris* contained a mixture of MS+PE with other components (AA, GER) showing little effect (Fig. 7C). PE was a component in the top nine traps and MS was found in the top five lure configurations, although MS by itself was not significantly different than the control traps.

The contrasts for *S. pyrastris* reflect the lure performance in that AA and GER had no significant effect either overall or for each individual comparison (Table 3). MS and PE both increased trap catch overall, and with most of the lure combinations. The comparison of MS versus the control showed no significant difference, but the other four comparisons that included PE resulted in significant increases in trap catch (Table 3).

Chrysoperla spp. The best performing traps for *Chrysoperla* spp. were AA+MS+PE or AA+GER+MS+PE. However, the AA+PE trap was not significantly different than the four-component trap (Fig. 7D), suggesting that for ease of use and cost reasons that it would be a reasonable trap for *Chrysoperla* spp. The contrasts for AA showed that it increases trap catch, but particularly so in the absence of GER, suggesting that an AA*GER interaction occurs. The effects of MS showed that it increases trap catch in general, although when using the Bonferroni corrections for the five different comparisons, no single combination was statistically significant (Table 3). The addition of GER did not reach statistical significance for any combination or overall comparisons. PE significantly increased trap catch in both the combinations present (Table 3).

Aphelinus mali (Haldeman) (Aphelinidae). This is a parasitoid of the woolly apple aphid (*Eriosoma lanigerum* (Hausmann)) and was caught in large numbers in the apple orchard (Quincy 1). The best trap was GER+PE, but the *lsd* test showed the top seven lure combinations were not significantly different (Fig. 8A) and all contained PE. From the standpoint of simplicity and cost, a single component trap with PE as the only lure would be reasonable for *A. mali*. The individual comparisons showed that the addition of AA, MS, or GER did not significantly affect trap catch. PE caused a significant increase overall, but that is the result of the difference between PE and the unbaited control trap; the difference in trap catch between MS and the MS+PE treatment were not significant (Table 3).

Aclastus spp. (Ichneumonidae). The ichneumonids as a group did not show statistical separation, but *Aclastus spp.* (some members of the genus parasitize spider eggs) was caught in large numbers in the walnut orchard in California and did exhibit significant lure effects. However, the *lsd* test revealed that the control traps were the second best treatment and were not significantly different than the best trap containing AP+PE (Fig. 8B). Thus, the statistical separation showed components that decreased trap catch. AA was a feature of the bottom four lures and PE was a component of the bottom six lures. PE by itself or in a binary mixture with AP were in the top grouping of traps, so that from the *lsd* test, PE may not be an issue except when combined with AA or MS. The overall contrasts showed that AA and MS both decreased trap catch, but there were no significant GER or PE effects (Table 3). Evaluation of the individual contrasts for AA showed that the PE versus AA+PE was highly significant, whereas none of the other contrasts reached statistical significance (Table 3). The MS effect was likewise associated heavily with the control and MS contrast, although all the MS contrasts resulted in negative Δ values.

Honeybees. Trap catch for honeybees was highest on the GER+MS+PE trap, but the top four lures did not separate out statistically (Fig. 8C). The top four lures all performed better than the control trap and all shared PE as a component, while the top 3 lures all contained GER as well. The contrasts for the different

components that AA and MS tended to reduce trap catch, while GER and PE tended to increase trap catch, however, none reached statistical significance (Table 3).

4. Discussion

Our studies show that a broad range of natural enemies can be readily trapped by plant volatile lures for monitoring purposes. The factorial analysis used in 2010 provides a much better understanding of how different component volatiles or combinations affect each taxon and helps guide the design of lure composition in traps to yield optimal activity and selectivity. The factorial analysis is manageable up to about four component volatiles, but logistically gets extremely difficult as each new component is added (the number of treatments needed is 2^n where n is the number of components tested). Thus in 2011, we opted for evaluating specific contrasts to investigate the effect of the addition of AA or MS to optimize blends that had previously been shown increase trap catch some of our more important natural enemy taxa. Some of the disparities where a particular component was found to be important in 2010, but not in 2011 (e.g., PE for honeybees or AA for *E. volucris*) are likely due to the greater power of the factorial design and also may have been modified by the stronger visual attraction of some groups to the yellow panels used in 2011.

While the exact combination of component volatiles that achieved the largest trap catch varied by taxon, some general trends did appear across the different experiments that we performed. Surprisingly, all the hymenopteran taxa with trap catch high enough to be analyzed responded strongly to PAA containing traps. While it is clear that we did not have enough different taxa/locations to suggest that it is a near universal lure for Hymenoptera, PAA is broadly found in floral volatiles (Knudsen *et al.*, 1993) and may be important to taxa that require floral resources for some aspect of their life history.

The general response of Hymenoptera to PAA was both exciting and troubling from the standpoint of monitoring for evaluation of BC. The large numbers of honeybees captured in the white delta traps

greatly complicated trap evaluations and was clearly an undesirable side effect. Switching to yellow or white panels or an orange delta trap greatly reduced honeybee catch (Jones, unpublished) to levels similar to those observed when no volatile lures were present (Knight and Miliczky 2004). Although not reported in this paper, we also observed large numbers of miscellaneous apoid bees (halictids, bumblebees and others) being caught in PAA-containing traps. Overall, the large numbers of Hymenoptera caught by PAA make it an exciting lure to evaluate for collection, and monitoring the abundance and diversity of Hymenoptera in an ecosystem and specifically as a great collecting tool for surveying honeybees for genetic diversity, health or presence in field situations.

The syrphid flies all responded strongly to PE (another common floral volatile), but there was significant variability related to their response to other lures, with *E. fumipennis* responding strongly to the presence of GER (+ trap catch) or AA (- trap catch) and *S. pyrastris* being relatively insensitive to either GER or AA. The effect of MS also varied among the different syrphid species, being important for *S. pyrastris*, but not for *E. fumipennis* or *E. volucris*. The syrphids highlight the potential issues associated with analyzing data at higher taxonomic levels, where the perceived efficiencies of different components may be affected by the relative abundance of the different species present within the larger taxonomic unit (e.g., family level) at different times of the season (Jones *et al.*, 2011; Rodriguez-Saona *et al.*, 2011). Evaluating how large taxonomic groups respond to a particular volatile is probably best performed only loosely and in situations where multiple lower level taxa are analyzed separately, data are from multiple locations and seasonal timings (e.g., as described above for the Hymenoptera), and where the results show similarity. However, even then, the conclusions must be viewed with caution because any logistically reasonable design will sample only a minute fraction of the possible species in the larger grouping (which may or may not be monophyletic).

Chrysoperla spp. responded positively to a broad range of different lure combinations, although many of the component volatiles tested appeared to generate a similar response so that additive effects were of

little benefit after 2-3 components were combined. Our best lure combinations contained most of the chemicals tested over the two-year period except for GER. Our two best combinations in 2010 were combinations of AA+AP and either PE or PAA, and in 2011 was AA+MS+PE. Our 2011 best trap was similar to the AA+MS+PAA trap reported to be optimal for catch of *C. carnea* in Europe (Toth *et al.*, 2006; Toth *et al.*, 2009). However, our tests did not include this particular combination. In the 2010 small factorial experiment, the AA+MS+PAA trap was tested, but unfortunately the number of *Chrysoperla* spp. caught was too low to analyze. However, examination of the raw trap catches did show that 42 of the 97 individuals caught (>2x the next best trap) were in the AA+MS+PAA trap suggesting that *Chrysoperla* spp. in Washington State respond well to that lure combination. The data from our 2010 large factorial did not include MS, but a comparison of the binary traps containing AA+PAA versus AA+PE showed there was no significant difference between them (Fig. 1C). Given the fact that *Chrysoperla* spp. is considered to be a species complex of up to six different cryptic species (Henry, 1985a; Henry, 1985b; Henry *et al.*, 2002) and that we could not distinguish between them on the traps, the lack of location effects (because of possible species differences) is somewhat surprising, although response to volatiles may be a highly conserved genetic trait.

The variability in response of different taxa to the different lure components opens the door to specific lures that minimize the trap catch of non-target natural enemies and the potential use of certain taxa of indicator species for evaluating BC potential. For example, review of table 3 shows that *Chrysoperla* spp. trap catch generally increases significantly with the addition of AA, whereas trap catch of *E. fumipennis* drops significantly. Similarly, GER tends to decrease *Chrysoperla* spp. catch while it increases *E. fumipennis* catch. When combined with various trap types and colors, selectivity of a trap to particular groups can be enhanced or reduced, which potentially makes monitoring for IPM purposes more reasonable. The larger size and relative ease of identification of both syrphids and lacewings together with their relative abundance in our systems suggest that they might be reasonable indicator groups for BC services in many western orchards.

Our studies also showed that, at least for the taxa we evaluated in our orchard systems, both MS and AA were rarely effective as lures on their own, but that in several situations that they acted synergistically when combined with other plant volatile lures. Previous studies have shown that MS alone can trap a broad range of natural enemies (James, 2003a; James, 2003b; James, 2005a; James, 2006; James and Grasswitz, 2005; James and Price, 2004) and a meta-analysis of 14 publications showed the effects of MS were significant for 41 of 91 different taxa and crop combinations, but in 50 of 91 were non-significant (Rodriguez-Saona *et al.*, 2011). While it is easy to speculate that the differences in MS effects between studies may be related to various factors such as release rates or taxa identified (either because it was limited or only at low taxonomic resolution), it may also be related to the interaction of MS with the complex volatile bouquet that occurs in whole plants (Dicke *et al.*, 2003; Snoeren *et al.*, 2010; Vet and Dicke, 1992). Thus, the effectiveness of the natural plant bouquet from crop plants may be synergized by the presence of MS from an independent lure. Rodriguez-Saona *et al.* (2011) also showed that exposure to a MS lure may induce changes in the volatile composition of natural plant bouquets after exposure, which could lead to apparent attraction to MS traps if the position of the traps is not constantly shifted from plant to plant.

The use of plant volatiles as monitoring tools are not as simple as the use of sex pheromones used for pest monitoring, but they are exciting potential tools for enhancing conservation BC in many agro-ecosystems. First, the environmental complexity and the range of potential volatiles competing for the attention of a natural enemy can vary markedly over time depending on stage of plant development. Thus, potentially the issue of differential effectiveness of lures over time needs to be considered. However, our studies reported elsewhere in this issue (Jones *et al.*, 2015) show that lures can be used to evaluate phenology as well for monitoring. Second, the degree of specificity of a particular volatile blend is clearly less than any sex pheromone, but there are possible combinations that limit trap catch of non-target natural enemies to help narrow the range of natural enemy taxa collected. At the same time, the broad activity patterns of

other blends can allow monitoring of a range of natural enemies in systems where the important taxa are poorly known. The lack of significant location effects in our 2010 large factorial and 2011 experiments, as well as the fact that many generalist natural enemies occur in a wide range of different ecosystems, suggest that the plant volatile lures may have much broader applicability than just the systems where their performance was originally evaluated.

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Table 1. Volatile components used for lures evaluated for natural enemy trap catch in western orchards 2010-2011.

Attractant	Bag	Release		Attractant Source
	thickness (mm)	Rate (mg/d) ¹	Volume (ml)	
Acetic Acid (AA)	0.1016	50.2	3.0	Acros Organics-222140010
Acetophenone (AP)	0.1016	58.7	3.0	Acros Organics-102410010
Geraniol (GER)	0.0381	9.9	1.0	SAFC Supply Solutions - W250708-1KG-K
Methyl Salicylate (MS)	0.1524	78.6	3.5	SAFC Supply Solutions -W274518-1KG-K
Phenylacetaldehyde (PAA)	0.1524	4.9	0.5	SAFC Supply Solutions -W287407-1KG-K
2-phenylethanol (PE)	0.0381	12.8	1.0	Sigma-Aldrich 77861-250ml

Table 2. Total number of individuals trapped for the more abundant taxa collected in the three experiments used for testing component volatile lures in different locations in California, Oregon, and Washington in 2010-2011. Bold values were not included in the statistical analysis for a particular taxon within an experiment.

Species	Family	Large Factorial 2010					Small Factorial 2010	2011 Trial		
		Yakima	Woodland	Quincy 1	Quincy 2	Hood River	Quincy	Yakima	Woodland	Quincy
		(apple)	(walnut)	(apple)	(apple)	(pear)	(apple)	(pear)	(walnut)	(apple)
<i>Apis mellifera</i>	Apidae	623	4566	17691	8038	3401	1067	9	20	228
<i>Chrysoperla</i> <i>spp.</i>	Chrysopidae	265	1170	289	679	133	97	1195	3098	245
<i>Eupeodes</i> <i>fumipennis</i>	Syrphidae	247	27	509	376	861	335	–	2266	272
<i>Eupeodes</i> <i>volucris</i>	Syrphidae	632	14	231	461	103	154	–	201	184
<i>Scaeva</i> <i>pyrastris</i>	Syrphidae	–	–	62	61	137	35	–	398	205
<i>Aphelinus</i> <i>mali</i>	Aphelinidae	–	–	–	–	–	–	–	–	3225
<i>Trechnites</i> <i>insidiosus</i>	Encyrtidae							934	–	–
	Ceraphronidae	–	–	–	–	–	–	–	–	366
	Eulophidae	485	19	2306	2123	306	605	37	–	–
	Figitidae	–	1009	722	1544	62	180	–	–	–
	Ichneumonidae	389	22	46	87	12	–	259	2528	–
<i>Aclastus</i> spp.	Ichneumonidae	–	–	–	–	–	–	–	1491	–
	Pteromalidae	0	1061	1331	598	116	594	30	–	126
	Scelionidae	122	–	1421	528	81	2053	240	–	1227

Table 3. Individual contrasts to test the effect of adding acetic acid (AA), methyl salicylate (MS), geraniol (GER), and 2-phenylethanol (PE), to various lures. For each factor tested (e.g., AA), the values are the differences between the trap catch scores of the lure components listed and the same components with the factor added (e.g., PE is tested against PE+AA).

Test	<i>Eupeodes volucris</i>	<i>Eupeodes fumipennis</i>	<i>Scaeva pyrastris</i>	<i>Chrysoperla spp.</i>	<i>Aphelinus mali</i>	<i>Aclastus spp. (Ichneumonidae)</i>	<i>Apis mellifera</i>
Lure effect ¹	0.0089	0.0008	< 0.0001	< 0.0001	0.009	< 0.0001	0.007
AA overall	-0.61	-2.51***	-0.04	2.91***	-2.00	-1.61***	-0.47
PE	-0.39	-1.19	-0.08	3.40**	-2.71	-3.22***	-0.65
GER+PE	-0.83	-2.93*	0.38	1.46	-2.20	-1.24	-0.61
MS+PE	-0.22	-2.99**	-0.35	4.51**	-0.26	-1.44	-0.17
GER+MS+PE	-1.05	-3.06*	-0.29	2.51	-0.47	-0.75	-0.51
MS overall	-0.13	0.09	1.13***	1.54*	0.22	-0.88*	-0.08
Control	-0.06	-0.29	0.21	0.47	2.07	-2.54*	-0.17
PE	-0.17	1.53	1.61***	1.53	1.12	-2.01	-0.74
GER+PE	-0.10	-0.02	1.75***	1.26	-0.142	-0.40	0.46
AA+PE	-0.00	-0.26	1.34**	2.64	1.23	-0.22	-0.26
AA+GER+PE	-0.32	-0.15	1.08*	2.32	-1.15	-0.09	0.56
GER overall	0.33	1.50*	-0.03	-0.77	1.48	-0.61	0.70
PE	0.64	2.74*	-0.22	0.20	2.8	-2.19	0.17
MS+PE	0.71	1.19	-0.09	-0.06	-1.20	-0.58	1.37
AA+PE	0.20	1.01	0.23	-1.74	3.30	-0.21	0.21
AA+MS+PE	-0.11	1.12	-0.03	-2.06	0.93	0.11	1.03
PE overall	1.02**	1.84**	1.37***	2.13***	3.76**	0.08	0.64
Control	1.08*	0.97	0.74*	1.64*	4.21*	-0.37	0.94
MS	0.97*	2.79 **	2.14***	2.69**	3.26	0.16	0.38

¹Significance level for the test of the lure effect in the ANOVA.

* Significant at ≤ 0.05 , ** significant at ≤ 0.01 , *** significant at ≤ 0.001 , all individual comparison values are adjusted using the Bonferroni correction; the overall factors (e.g., AA overall) are not corrected. Values without asterisks are not statistically significant.

Figure

Figure 2

Figure3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8