5042

Green Chemistry Letters and Reviews
Vol. 1, No. 4, December 2008, 205–217

Taylor & Francis
Taylor & Francis Group

RESEARCH LETTER

Environmentally regulated abiotic release of volatile pheromones from the sugar-based oral secretions of caribflies

Spencer S. Walse[a,b]*, Hans T. Alborn[b] and Peter E.A. Teal[b]

[a]*United States Department of Agriculture, Agricultural Research Service, San Joaquin Valley Agricultural Sciences Center, 9611 S Riverbend Ave, Parlier, CA, 93648 USA;* [b]*United States Department of Agriculture, Agricultural Research Service, Center for Medical, Agricultural, and Veterinary Entomology, 1700 SW 23rd Dr, Gainesville, FL, 32604 USA*

(Received 15 August 2008; final version received form 19 January 2009)

We report an abiotic mechanism for the emission of volatile insect pheromones that is controlled by environmentally induced change in the physicochemical properties of the sugar-based release matrix. Male *Anastrepha suspensa* (Loew) (caribflies) mark mating sites on leaf surfaces by depositing oral secretions that contain sugar, as well as, γ-hydroxy acid and γ-lactone forms of the diastereomeric aggregation pheromones, epianastrephin and anastrephin. The γ-hydroxy acids extend emission over many days via aqueous equilibrium with the thermodynamically less preferred, but more volatile, γ-lactones (~100:1). A kinetic model, which supports a γ-lactone diffusion-limited rate, was generated and tested by measuring the effect of temperature and humidity under fixed and ambient atmospheric inputs, respectively. Results show that pheromone release from the markings occurs with a periodicity that parallels relative humidity and complements the daily pattern of the caribflies' reproductive and aggregative activity. This study provides an example of a physicochemical-based inter-organism communication strategy that has been mechanistically linked to the abiotic environmental processing of volatile chemical signals. The exploitation of this natural connectivity will spur environmentally sustainable chemistries, particularly pheromone-based alternatives to insecticide application.

Keywords: controlled release; pheromones; tephritid fruit flies; environmental processing; γ-lactones

Downloaded By: [University of California, Berkeley] At: 01:55 13 February 2010

Introduction

One strategy for the development of environmentally sustainable chemical technologies involves the mimicry of processes that mediate natural product interaction with the environment. Elucidating the fate and transport of semiochemicals, natural products that function as signals in inter-organism messages, can be particularly useful in this capacity because their interaction with biotic, as well as abiotic, environmental factors is intrinsic to their evolved utility. Natural biotic mechanisms of semiochemical release, attenuation, and preservation have been studied extensively and are often influenced by environmental parameters (e.g. photoperiod, temperature, and humidity); yet, corresponding literature regarding abiotic mechanisms is lacking. This is curious because understanding natural abiotic processes that influence semiochemical "transmission" will benefit many chemical technologies intended for environmental application.

Pest management practices based on the exploitation of volatile semiochemicals produced by insects provide a poignant example of such technologies. Their utility as sustainable species-specific alternatives to broadcast insecticide application is limited, in many cases, due to an ineffective abiotic release of semiochemicals into the environment. To date, almost all of the semiochemicals employed in this context are naturally released "biologically" by insects directly into air as a function of environmental factors; this type of mechanism is difficult to replicate. In contrast, volatile semiochemicals can also be released "abiotically" from materials deposited by insects for the purpose of marking a location for revisitation. These natural abiotic mechanisms for semiochemical release, which are just beginning to be characterized, are easier to formulate effectively for use in pest control programs.

For example, in 2002 ~500,000 lbs. of organophosphorus insecticides were applied to citrus, in part to counteract tephritid fruit fly infestation (*1*) that has

*Corresponding author. Email: spencer.walse@ars.usda.gov

ISSN 1751-8253 print/ISSN 1751-7192 online
This work was authored as part of Contributor's official duties as an employee of the United States Government and is therefore a work of the United States Government. In accordance with 17 U.S.C. 105 no copyright protection is available for such works under U.S. law.
DOI: 10.1080/17518250902758895
http://www.informaworld.com

the potential to damage fruit worth ~$7 billion to the US annually (*2*). To reduce non-target health risks, naturally produced sexual attractants (i.e. pheromones) can be used for controlling populations of certain tephritid species. In Florida, the Caribbean fruit fly (caribfly) (*Anastrepha suspensa* (Loew); (Diptera: Tephritidae)) represents a serious threat to citrus commodities and nine volatile compounds released by males have been identified that presumably act as pheromones (*3–6*). Yet, effective lure and trap systems based on these semiochemicals are still lacking due, predominately, to an inability of rubber septa "bleed" based (*7–10*), capillary-based (*11*), and membrane-based technologies to mimic the natural ratios and release rates of the pheromone blend over prolonged periods (*12–14*).

In nature, male *Anastrepha suspensa* (Loew) (caribflies) deposit sugar-based oral secretions (OS) on the underside of plant leaves and these "marked" leaves are frequented day after day by both sexes in the wild (*15,16*). This suggested that the aggregation of caribflies, the preliminary step in a complicated lek mating system, could result from pheromones that are released abiotically into air for prolonged periods from OS after its deposition (*10,15,16*). Recent bioassays, in fact, demonstrated that male OS is highly attractive to conspecific males and virgin females (*16*). Two diastereomeric *trans*-fused γ-lactones, epianastrephin (ES) and anastrephin (AS) (collectively ES/AS) (*3,16,17*), were identified as aggregation pheromones since they emanated for weeks from male OS and proved to be critical for attraction (Figure 1(a)).

In this manuscript, we describe the mechanism of ES/AS release from OS and how it is affected by abiotic environmental parameters. To uncover its molecular-level underpinnings, the physicochemical phenomenon that influence the volatile emission (i.e. liquid to air transfer) of these pheromones were investigated: the equilibrium distribution of ES/AS between their γ-lactone and γ-hydroxy acid (HA) forms, the kinetics of inter-conversion between the forms, respective Henry's Law constants, and molecular diffusion in OS. A kinetic model, which supports a γ-lactone diffusion-limited rate, was generated by measuring the effect of temperature and humidity, under fixed (i.e. controlled) laboratory inputs; it was then tested using ambient (i.e. uncontrolled) atmospheric inputs within model ecosystems that simulated natural conditions. We also briefly explain how the mimicry of this natural abiotic mechanism for aggregation pheromone release has significant potential to control caribfly populations since it appears to be an integral component of their reproductive strategy.

Results and discussion

Chemical composition of oral secretions (OS)

The sugar loading and composition in freshly collected male and female OS (pH 5.5 ± 0.3; $\bar{x} \pm s$) was determined to be 33.2 ± 2.1 wt.% in solution at a ~ D-glucose:2 D-fructose:sucrose ratio. The concentrations (grand mean ± SE, $n = 18$) (*18*) of the lactone and acid forms of ES/AS in pooled samples of male OS were 11 ± 3 and 24 ± 4 ng/μL, respectively. The ratio of ES:AS and ES HA:AS HA, ~2.5:1, was also observed in volatile collections and other abiotic studies on ES/AS distribution (*19*). None of these pheromone components were detected in female OS.

pKa determination

The pKa for ES HA and AS HA were 5.0 ± 0.1 and 5.1 ± 0.3, respectively. These values were determined as outlined in Harris (*20*) using potentiometric titration ($n = 3$) of 0.9 mM ES/AS HC (sodium salt) with 1 mM trichloroacetic acid in 10% (v/v) isopropanol and $u \approx 0.01$ M (NaCl) at 25°C.

Inter-conversion of acid and lactone epianastrephin/anastrephin (ES/AS) forms

Three structural variants of intra-molecular esters, β-, γ-, and δ-lactones, are particularly common in semiochemicals. A unique abiotic feature of these lactones, coincident with Baldwin's ring-closure rule (*21*), is that they exist in equilibria with relatively hydrophilic β-, γ-, and δ-hydroxy acids, respectively, when in aqueous systems. Specifically, the γ-lactone moiety of ES/AS was converted into a γ-hydroxy acid (HA) and corresponding carboxylate under the conditions: $[ES/AS] < [OH^-] < [buffer]$. Although there are rate constants associated with buffer-catalyzed (k_{NaHCO_3}), H_2O-catalyzed (k_{H_2O}), and acid-catalyzed ($k_{H_3O^+}$) hydrolysis, their contributions were minimal in this system as suggested by previous work (*22,23*). Acid-catalyzed lactonization (k_{LAC}) of γ-hydroxy acids to form *cis*-fused γ-lactones can occur rapidly and often the γ-lactone (i.e. closed ring) form is largely favored at equilibrium (*23*). However, these are not characteristic features of analogous *trans*-fused systems (*24*); ES/AS concentrations over 30 days at pH 5.5 comprised only ~1% of solutions fortified with ES/AS HA over the range 20–40°C.

Downloaded By: [University of California, Berkeley] At: 01:55 13 February 2010

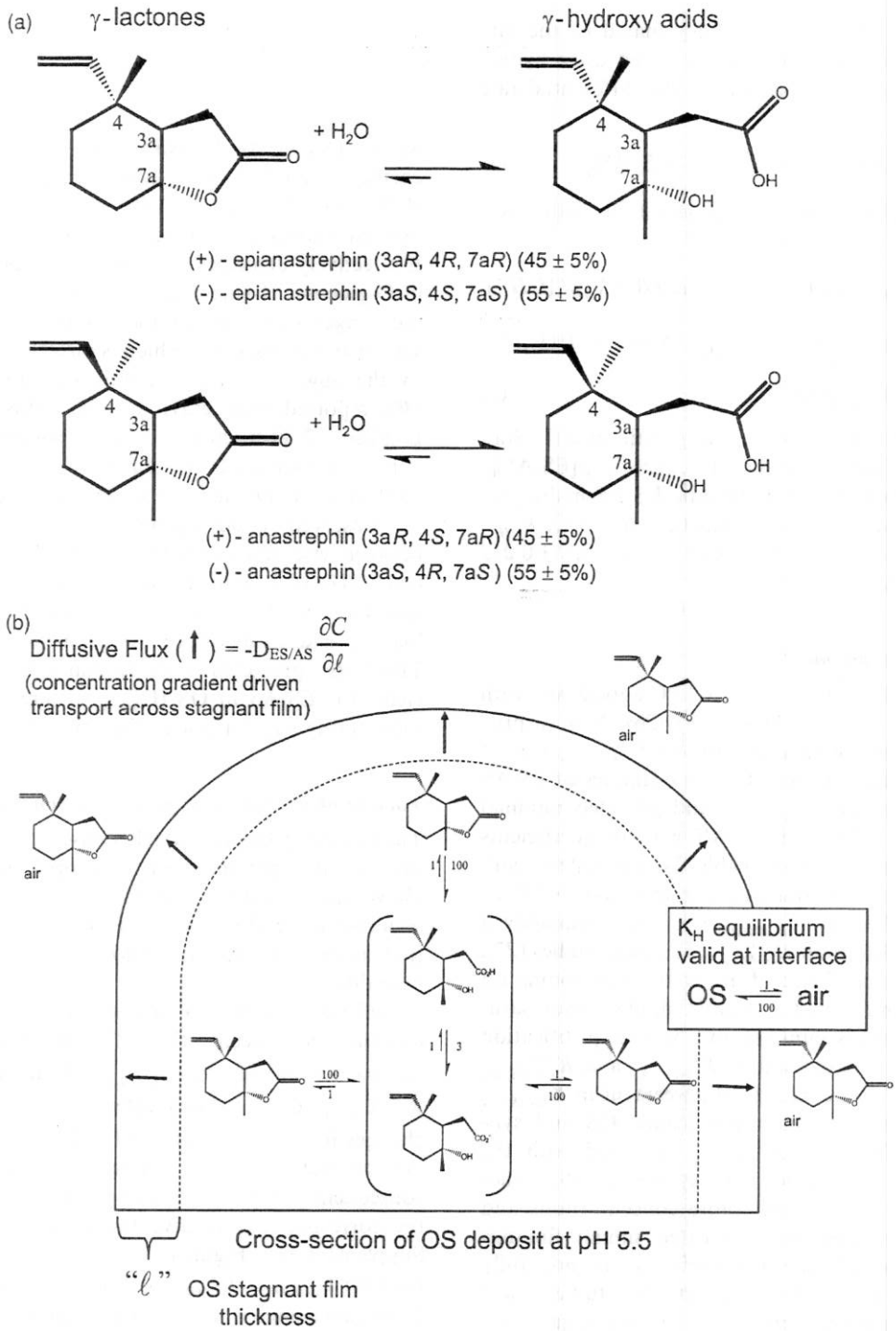

Figure 1. (a) Diasteromeric *trans*-fused γ-lactone and γ-hydroxy acid forms of aggregation pheromones, present in a ratio of ~2.5 epianastrephin to anastrephin, occur in the sugar-based OS of male caribflies. (b) This study provides evidence to support a diffusion-limited rate of epianastrephin and anastrephin emission from OS that is driven by aqueous speciation between the precursory γ-hydroxy acid forms and their thermodynamically less preferred (~100:1), but relatively volatile, γ-lactone equivalents (illustrated here for (+)-epianastrephin).

Downloaded By: [University of California, Berkeley] At: 01:55 13 February 2010

Accordingly, ES/AS loss was dominated by the rate associated with specific base-catalyzed ester hydrolysis (k_{OH}^-) and is expressed by the differential rate equation:

$$-d[ES/AS]_{aq}/dt = k_{HY\text{-}ES/AS}[ES/AS]_{aq} \quad (1)$$

where the observable rate constant of hydrolysis, $k_{HY\text{-}ES/AS}$ (s^{-1}), is defined as (*22,25*):

$$k_{HY\text{-}EA/AS} = k_{H_3O}^+[H_3O^+] + k_{H_2O}[H_2O] + k_{OH}^-[OH^-] + k[NaHCO_3] - k_{LAC}[ES/AS\ HA][H_3O^+] \cong k_{OH}^-[OH^-] \quad (2)$$

Experimental data support the kinetic model (Supplementary Table 1); plots of ($\ln[ES/AS]_t/[ES/AS]_0$) versus time were linear, indicating ES/AS hydrolyses followed *pseudo* first-order kinetics. At 40°C, k_{OH}^- (s^{-1}) had values of 0.108 ± 0.013 and 0.127 ± 0.012 for ES and AS, respectively.

Henry's law experiments

Measured values of $K'^{sat}_{H\text{-}ES/AS}$ at 25°C agreed well with an estimated value (1.33×10^{-2}) (*26*). Not surprisingly, the effect of temperature on $K'^{sat}_{H\text{-}ES/AS}$ was of similar magnitude to its effect on estimates of ES/AS vapor pressure ($P^{\circ}_{ES/AS}$) (*26*) and reflected minimal variation in calculated ES/AS activity coefficients ($\gamma^{sat}_{w\text{-}ES/AS}$) (Supplementry Table 2). Consistent with their acid to lactone equilibrium distribution ($\sim$100:1), measured values of $K'_{H\text{-}ES/AS}$ were approximately a factor of 100 less than $K'^{sat}_{H\text{-}ES/AS}$. Previous studies (*27*), suggest the dependence of air–water partitioning on solute concentration (i.e. within dilute versus saturated solutions) is minimal and provide justification for the direct comparison of $K'_{H\text{-}ES/AS}$ and $K'^{sat}_{H\text{-}ES/AS}$ in this study. There was good agreement in $K'^{sat}_{H\text{-}ES/AS}$ measurements obtained using female OS and synthetic OS (Table 1). $K'^{sat}_{H\text{-}ES/AS}$ increased with the temperature of female and synthetic OS; other studies, over comparable solute concentrations and temperature ranges, have reported similar findings (*28,29*). Although the mechanism(s) is not fully understood, many structure–activity studies have explored the positive correlation between sugar loading and the air to water distribution of esters (*28–34*); our observations support this trend (Supplementary Figure 1).

Fickian diffusion in oral secretions (OS)

The relationship in OS between molecular diffusivity, viscosity (μ), and temperature can be generalized by the Stokes–Einstein equation:

$$D_{ES/AS} = \frac{k_B T_{OS}}{6\pi r \mu_{OS}} \quad (3)$$

where $D_{ES/AS}$ is ES/AS's translational diffusion coefficient ($cm^2\ s^{-1}$), k_B is the Boltzmann constant (1.38×10^{-23} kg $m^2\ s^{-2}\ K^{-1}$), and r is the hydrodynamic radius of "spherical" ES/AS ($\sim$4.47 Å) (*35*). It effectively describes the diffusion of ester volatiles in D-glucose, D-fructose, and sucrose solutions over the ranges examined in this study (*36–40*). Citing similarities in viscosity, which is directly proportional to the sugar loading, Chandrasekaran and King (*30*) reported that ethyl acetate diffusion in $\sim$1 D-glucose:2 D-fructose:1 sucrose solutions reasonably approximated its diffusion in sucrose (or D-glucose, or D-fructose) solutions alone. Likewise, we observed good agreement (<10% variation) between viscosity estimates based solely on sucrose (μ_{SU}) (*41*) and viscosity measurements of OS (μ_{OS}) and its synthetic analogs (μ_{SA}) at a given sugar loading and temperature (Supplementary Table 3). Therefore, predicted translational diffusion coefficients for ES/AS in OS ($D'_{ES/AS}$) were obtained by substituting μ_{SU} for μ_{OS} in Equation 3.

Volatile pheromone emission: fixed conditions

The following kinetic model is based on an assimilation of the physicochemical properties described above and was developed to explain measurements of airborne ES/AS, released from OS, within VCCs that permitted tunable temperature and absolute humidity.

OS functioned as a humectant due to its sugar content; the concentration of water in air ($C_{a\text{-}H_2O}$) relative to that in OS ($C_{OS\text{-}H_2O}$) at equilibrium (i.e. $K = C_{a\text{-}H_2O}/C_{OS\text{-}H_2O}$) was affected, as indicated by changes in OS volume (from 10 μL), by temperature and absolute humidity (Table 2). OS volume, and consequently, OS sugar loading were proportional to the corresponding relative humidity (%) (Table 2, Supplementary Figures 2–5). The equilibrium between $C_{a\text{-}H_2O}$ and $C_{OS\text{-}H_2O}$ was established within 2 h (Supplementary Figure 6); accordingly, ES/AS concentrations measured >2 h after deposition were utilized in model development. There was minimal difference in the volatile emission of ES/AS from either supplemented female or male OS at air flows of 140, 315, 480, or 660 cm^3/min.

The flux (mol $cm^{-2}\ s^{-1}$) of ES/AS from female OS supplemented with ES/AS could be described by a stagnant boundary model under liquid-film control and the partial differential equation (*42,43,44*):

Downloaded By: [University of California, Berkeley] At: 01:55 13 February 2010

Table 1. Air to water equilibrium distribution of ES/AS, reported as $K'^{sat}_{H\text{-}ES/AS}$ ($\times 10^2$), was affected by sugar loading and temperature.

	Sugar loading (wt.%) at ~ D-glucose:2 D-fructose:sucrose ratio					
	Female OS	Synthetic OS (0.01 M $NaHCO_3$, pH 5.5)				
Temperature (°C)	~33	25	33	45	55	65
20	0.8±0.3	0.7±0.4	0.9±0.2	1.2±0.4	1.4±0.2	1.8±0.4
25	1.5±0.3	1.4±0.5	1.8±0.4	2.0±0.3	2.5±0.5	3.1±0.3
30	2.0±0.4	1.8±0.4	2.2±0.3	3.4±0.2	3.5±0.5	5.4±0.5
35	2.9±0.6	2.8±0.6	3.2±0.3	4.5±0.5	5.0±0.4	6.6±0.6
40	4.8±0.5	4.5±0.3	5.0±0.4	5.7±0.5	6.2±0.6	7.3±0.4

Table 2. OS functioned as a humectant due to its sugar content, that is, the equilibrium distribution of water between air and OS was affected, as indicated by changes in OS volume (from 10 μL), by temperature and absolute humidity over the ranges of this study.

		Absolute humidity, C_{a-H_2O} (mM)			
Temperature (°C)		0.67±0.2	0.77±0.2	0.85±0.2	0.92±0.3
20	Relative humidity (%)[a]	70	81	89	96
	OS volume (μL)	7.0±0.4	9.2±0.3	11.8±0.4	16.1±0.6
	OS–SA (cm^2)[b]	0.33	0.34	0.45	0.58
	wt. (%)[c]	41.5	35.1	29.6	23.6
	$\chi_{w\text{–}OS}$	0.94[d]	0.94[d]	0.95[d]	0.95[d]
	α ($\times 10^5$ cm s^{-1})[e]	5.8±0.5	8.1±0.8	13.3±0.8	18.3±0.9
25	Relative humidity	52	60	67	75
	OS volume	5.5±0.4	6.6±0.5	8.7±0.4	12.1±0.4
	OS–SA	0.24	0.28	0.43	0.56
	wt. (%)	47.5	43.0	36.4	29.1
	$\chi_{w\text{–}OS}$	0.93	0.94	0.94	0.95
	α ($\times 10^5$ cm s^{-1})	3.6±0.3	5.6±0.6	9.2±0.6	15.3±0.7
30	Relative humidity	39	45	51	58
	OS volume	3.0±0.2	4.2±0.2	6.4±0.4	9.5±0.5
	OS–SA	0.19	0.23	0.28	0.44
	wt. (%)	62.4	54.7	43.7	34.3
	$\chi_{w\text{–}OS}$	0.92	0.93	0.94	0.95
	α ($\times 10^5$ cm s^{-1})	1.9±0.2	3.1±0.4	5.1±0.3	12.5±0.6
35	Relative humidity	30	35	39	42
	OS volume	2.0±0.5[f]	3.0±0.3	3.5±0.3	4.5±0.2
	OS–SA	0.18	0.19	0.19	0.26
	wt. (%)	70.6	62.4	58.7	52.5
	$\chi_{w\text{–}OS}$	0.90	0.92	0.92	0.94
	α ($\times 10^5$ cm s^{-1})	1.9±0.2	2.2±0.2	2.5±0.2	3.3±0.3
40	Relative humidity	23	28	30	34
	OS volume	1.0±0.5[f]	1.8±0.5[f]	2.0±0.5[f]	2.8±0.4
	OS–SA	0.17	0.17	0.18	0.18
	wt. (%)	83.5	73.8	66.5	64.4
	$\chi_{w\text{–}OS}$	0.86	0.90	0.91	0.92
	α ($\times 10^5$ cm s^{-1})	0.7±0.2	1.4±0.2	1.9±0.1	2.5±0.2

[a] $100 \times C_{a\text{-}H_2O}/C^{sat}_{a\text{-}H_2O}$, where $C^{sat}_{a\text{-}H_2O}$ varies with temperature.
[b] Error = ±0.07 cm^2 (see Supplementary Figure 4).
[c] Sugar loading (wt.%) in OS at a ~ D-glucose:2 D-fructose: sucrose ratio.
[d] Moles water/moles water + moles total carbohydrates + moles ES/AS.
[e] Corresponds to release from ES/AS supplemented female OS (10 μL deposit).
[f] Determined by extrapolation of linear least-squares analysis of data with OS volume 5.5–2.75 uL versus relative humidity (%) (see Supplementary Figure 5).

Downloaded By: [University of California, Berkeley] At: 01:55 13 February 2010

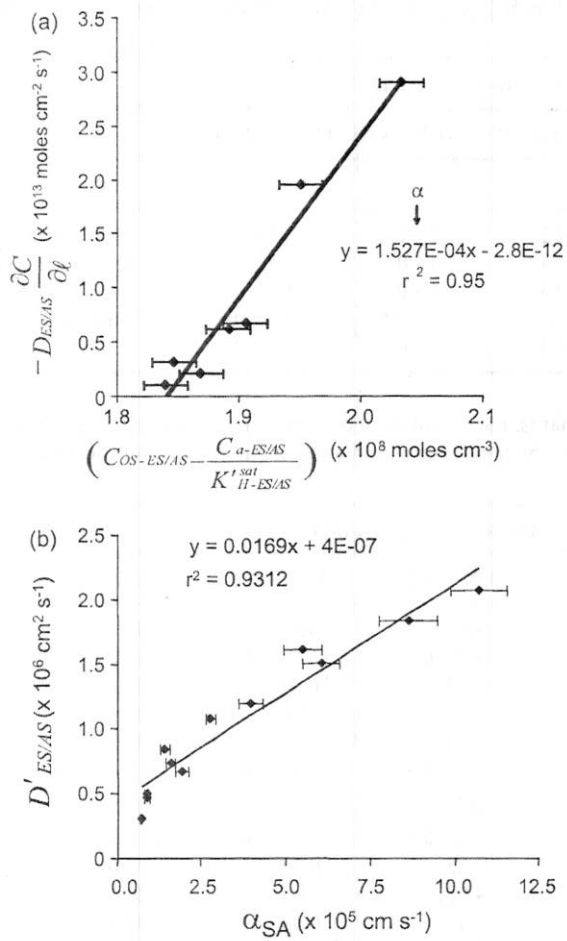

Figure 2. (a) The linear relationship (correlation coefficient: $r^2 = 0.95$) between the flux of ES/AS and $\left(C_{OS\text{-}ES/AS} - \frac{C_{a\text{-}ES/AS}}{K'^{sat}_{H\text{-}ES/AS}}\right)$ at 25°C and absolute humidity of 0.92 mM. A similar relationship, the slope of which is termed α, was obtained over the investigated ranges of temperature and humidity and supports a diffusion-controlled liquid to air partitioning (i.e. volatilization) of ES/AS (*44*). (b) The linear relationship (correlation coefficient: $r^2 = 0.92$) between α_{SA} and $D'_{ES/AS}$ in this study; the slope obtained by linear least-squares analysis of the data represent ℓ/L.

$$\text{Flux (F)} = -D_{ES/AS}\frac{\partial C}{\partial l} = \alpha\left(C_{os\text{-}ES/AS} - \frac{C_{a\text{-}ES/AS}}{K'^{sat}_{H\text{-}ES/AS}}\right) \quad (4)$$

where ℓ (cm) is the thickness of the OS stagnant film, $C_{OS\text{-}ES/AS}$ and $C_{a\text{-}ES/AS}$ are the respective ES/AS concentrations (mol cm^{-3}) in OS and air, and α is a proportionality constant (cm s^{-1}) that is defined as (*44*):

$$\alpha = \frac{L\, D_{ES/AS}}{\ell} \quad (5)$$

Note that under the conditions where L, a dimensionless parameter, and ℓ are established, α represents the transfer velocity of ES/AS through the stagnant layer, v_{OS} (cm s^{-1}).

Experimental data support this kinetic model and suggests the release rate of ES/AS from supplemented female OS results from diffusion-limited liquid to air transfer that is fast relative to ES/AS hydrolysis at pH 5.5 ($t_{1/2}$@40°C ≅ 12–20 × 10^3 d); plots of $\left(C_{OS\text{-}ES/AS} - \frac{C_{a\text{-}ES/AS}}{K'^{sat}_{H\text{-}ES/AS}}\right)$ versus $\left(-D_{ES/AS}\frac{\partial C}{\partial \ell}\right)$ were linear in all cases (Figure 2(a)). Relative humidity affected the slope (α) obtained by a linear least-squares analysis, independent of its effect on $K'^{sat}_{H\text{-}ES/AS}$ (Table 1 and 2, Supplementary Table 2, Supplementary Figures 1 and 7).

To further isolate the role of diffusion in the relation between α and relative humidity, we normalized measured fluxes with the corresponding OS surface area (SA) (see Table 2) to obtain a SA-corrected description of the release rate of ES/AS from OS (mol s^{-1} or ng h^{-1}):

$$\text{release rate/OS SA} = \alpha_{SA}\left(C_{OS\text{-}ES/AS} - \frac{C_{a\text{-}ES/AS}}{K'^{sat}_{H\text{-}ES/AS}}\right) \quad (6)$$

Figure 2(b) shows the direct linear relationship (correlation coefficient: $r^2 = 0.92$) between α_{SA} and $D'_{ES/AS}$ for data above 40% relative humidity, since relative humidity lower than this are atypical within the geographic distribution of caribflies (*45*). According to Equation 5, the slope obtained by linear least-squares analysis of the data represent ℓ/L. Evaluation of equations developed in Crank (*44*) to describe diffusion in a cylinder and (hemi)sphere under conditions of "surface evaporation," yield L approximations of two and three, respectively, for this system. These L values correspond to an overall OS stagnant film thickness (ℓ) of ~0.04 cm, a reasonable estimate based on values for water ($\ell_{H_2O} \approx 0.05$–0.005 cm) (*42*).

Our data indicate the diffusion-limited release rate of ES/AS from OS is sensitive to changes in temperature and humidity to the extent that their daily fluctuation elicits a marked effect. However, even when "dry" air at 40% relative humidity was maintained, there was >98% recovery within 15 h after deposition from 10 μL of female OS supplemented with ES/AS at concentrations naturally found in male OS. Since ES/AS aggregation pheromone release from natural male OS occurs over many days, or weeks, we investigated whether the

Downloaded By: [University of California, Berkeley] At: 01:55 13 February 2010

Downloaded By: [University of California, Berkeley] At: 01:55 13 February 2010

extension of volatile emission was attributable to the γ-hydroxy acid forms of ES/AS, which occur at nearly twice the concentration of the γ-lactone forms in fresh OS.

When ES/AS release from female OS supplemented with ES/AS HA was experimentally determined and measured values of $K'_{H\text{-}ES/AS}$ were substituted into Equation 4 for $K'^{sat}_{H\text{-}ES/AS}$, α (or α_{SA}) was reduced ~100-fold in comparison to identical conditions with ES/AS supplemented OS (Supplementary Figure 8). This reduction corresponded with ~100-fold increase in the duration of ES/AS volatile emission from OS. At relative humidity levels <75%, these studies were impeded by the time course required to collect seven data points for the kinetic model; predicted release rates indicate sampling would be required for ~12,000–120,000 h in these instances, well over (>10 ×) the average caribfly lifespan.

The reflection (~1:1) of $K'_{H\text{-}ES/AS}$ on α_{SA} provides additional evidence for this kinetic description and is consistent with a ES/AS release mechanism driven by aqueous equilibrium between γ-hydroxy acids, corresponding carboxylates, and their thermodynamically less preferred (~100:1), but relatively volatile, γ-lactone equivalents (Figure 1(b)). In OS, this speciation was apparently only minimally influenced by humidity-induced changes in the relative concentrations of involved species (χ_{w-OS} >0.95) (see Table 2).

We were able to mimic this natural abiotic mechanism by supplementing female OS with γ-hydroxy acid and γ-lactone forms of ES/AS at concentrations matching those in males, as the release from 10 μL of the "composite" and male OS were consistent (Figure 3). When the first data points (<24 h), which represent release derived primarily from the γ-lactone forms of ES/AS initially present in OS, are omitted from plot (b), note the ~100-fold reductions in α values compared to OS supplemented with ES/AS only at 75% relative humidity (see Figure 2(a)).

Volatile pheromone emission: simulated natural conditions

The predictive kinetic description of volatile pheromone emission was tested in model ecosystems that were designed to simulate natural conditions, particularly with respect to temperature and humidity inputs. A 24 h time course was chosen to isolate the release of volatile ES/AS that occurs as a result of "fresh" OS deposits. Consequently, impacts on the ES/AS pheromone emission resulting from ES/AS HA and the colonization of OS by phylloplane microbes were negligible.

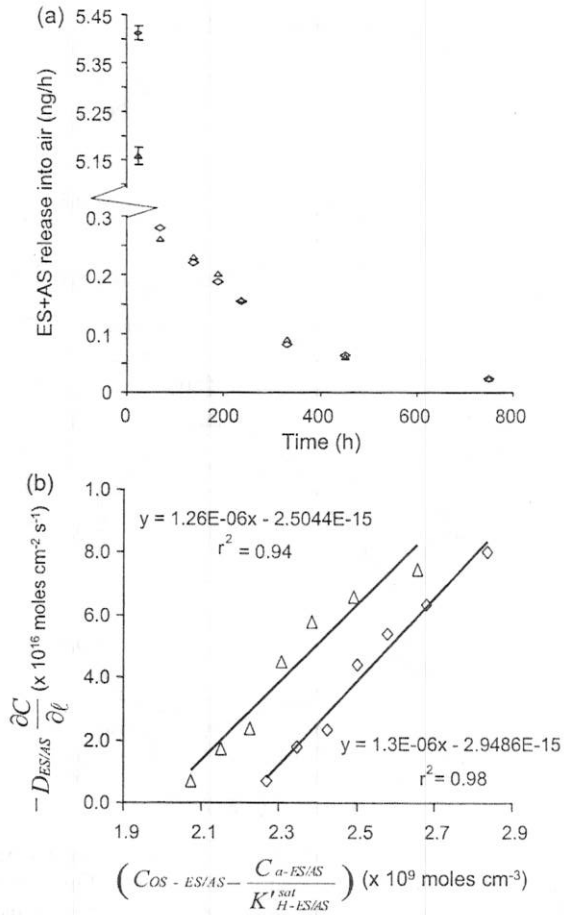

Figure 3. (a) When female OS (10 μL) was supplemented with γ-lactone (11 ng/uL) and γ-hydroxy acid (24 ng/uL) forms of ES/AS at concentrations matching those males, release at 75% relative humidity from the "composite" (◇) and male (△) OS were consistent. (b) Note the ~100-fold reductions in α relative to that in Figure 2(a), when OS was supplemented under identical conditions with just the γ-lactone forms of ES/AS.

Within the model ecosystems, predicted ES/AS diffusion in OS ($D'_{ES/AS}$) paralleled relative humidity and was inversely related to temperature (Figure 4(a)). ES/AS emission from OS (ng/h) was measured and also predicted by solving Equation 6 with the average temperature and relative humidity values over the respective dates (A: 31°C, 58%; B: 19°C, 68%); between comparisons, initial ES/AS concentrations were similar (A: ~3 μg; B: ~0.5 μg) and OS SA was assumed to be equal. The deviation between observed and predicted release fluctuated with the periodicity of $D'_{ES/AS}$ (Figure 4(b)). We have interpreted the results to mean that under ambient atmospheric inputs of temperature and humidity, just as under fixed inputs, ES/AS volatile emission from OS occurs with a diffusion-limited rate.

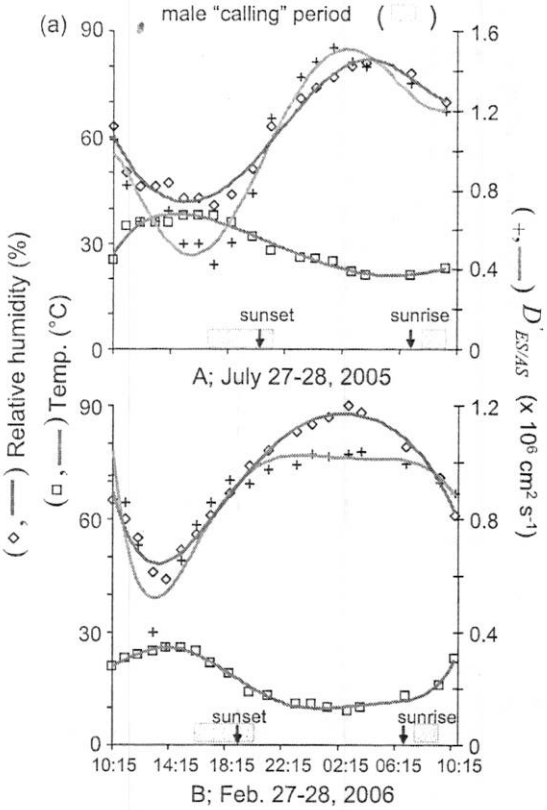

Figure 4(a). The predicted diffusion coefficients of ES/AS in OS ($D'_{ES/AS}$) relative to environmental parameters within the model ecosystems. Males biologically synthesize and release aggregation pheromones during "calling" periods (▭).

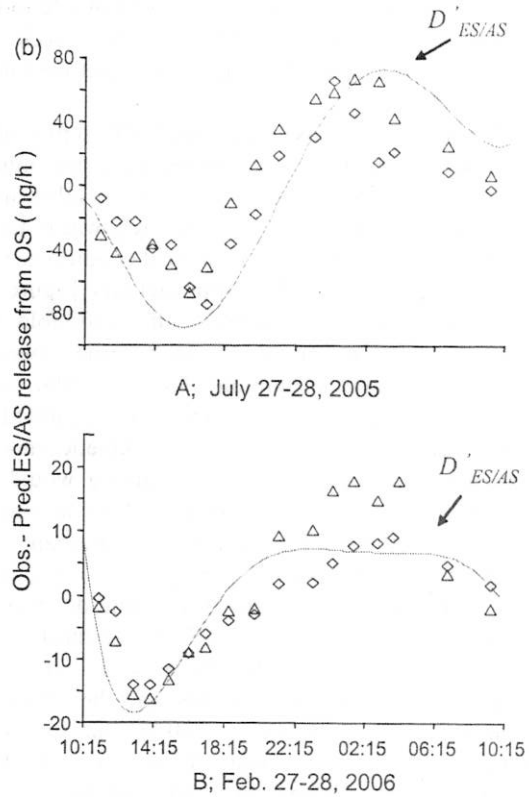

Figure 4(b). OS deposition from 100 male caribflies occurred "naturally" onto glass in A (△, rep. 1; ◇, rep. 2) and male OS was mechanically deposited (10 × 10 μL) onto the underside of loquat leaf (△) or glass-slide (◇) surfaces within B. The deviation between observed and predicted release of volatile ES/AS from OS, based on solving Equation 6 with time-course average temperature and relative humidity values, fluctuated with the periodicity of $D'_{ES/AS}$.

It is interesting to note that within model ecosystem B there appears to be a nocturnal enhancement of ES/AS pheromone release from OS deposited on the underside of "natural" leaf surfaces relative to glass. Given the topographically heterogeneous microstructure of the loquat leaf epidermis relative to the surface of glass, OS would be expected to have a larger effective SA when deposited on leaves, facilitating additional mass transport of atmospheric moisture and ES/AS through the leaf-OS interfacial region. Non-atmospheric biological water supplies to OS not applicable to glass, such as transpiration and/or guttation (*46*), could also function to decrease OS sugar loading and increase ES/AS emission from OS on a leaf surface. However, they are not likely because data on gas exchange and leaf-water content at mid-day relative to pre-dawn indicate that loquat stomata are closed at night (*47*).

The pattern of caribfly reproductive activity must be examined to appreciate the ecological utility of this environmentally regulated abiotic strategy for aggregation pheromone release. Mature male "calling" behavior occurs during two periods each day, beginning ~30 min after sunrise and ~3 h prior to sunset (see Figure 4(a)) (*10,46–48*). ES/AS biological synthesis and release, OS deposition, and female attraction to lek sites is known to occur during these periods, although they are more pronounced in the afternoon when mating occurs. ES/AS release from a single male (~1000 ng/h) is considerably larger than the release from OS deposited by 100 males (~300 ng/h); thus, there is a strong potential for periods of biological release to overwhelm abiotic release from OS, particularly that originating from remote locations. Our data indicate, however, that the maximum in predicted diffusion ($D'_{ES/AS}$) occurs between "calling" periods to provide release of ES/AS from deposited OS markings at night and in the early morning when relative humidity is high. Interestingly,

Downloaded By: [University of California, Berkeley] At: 01:55 13 February 2010

the diel periodicity of this abiotic release coincides with male attraction to lek sites, which is known to occur around sunrise in the field (*49,50*).

Time-resolved bioassays in flight tunnel model ecosystems, which would function to bridge the mechanistic data presented here and the field observations, are needed to confirm that male attraction to male OS occurs in the early morning, before they begin biological ES/AS release to presumably establish mating territories. Nevertheless, our results strongly suggest that the environmentally regulated abiotic release of aggregation pheromones is a critical aspect of the inter-caribfly communication strategy because the abiotic environmental processing of aqueous equilibria is what that regulates the volatilization of ES/AS from male OS.

Downloaded By: [University of California, Berkeley] At: 01:55 13 February 2010

Experimental

General

The chemical characterization, isolation, and synthesis of ES, AS (*3*), and their respective γ-hydroxy acids (ES HA and AS HA) were as reported in Walse et al. (*17*). Barnstead E-pure™ water (18 MΩ cm) was used for solutions. All other chemicals were obtained from commercial sources unless otherwise noted. Gas chromatography – ion trap mass spectrometry (GC–MS) and high performance liquid chromatography – electrospray ionization mass spectrometry (HPLC–ESIMS) retention times and spectra were used for chemical verification (Supplementry Table 1). Specifics of the analytical methodology, reported previously (*17*), are briefly described in supporting online material.

Inter-conversion of acid and lactone epianastrephin/anastrephin (ES/AS) forms

A series of 0.01 M buffers, set to ionic strengths of $u = 0.1$ M with NaCl, were adjusted with 0.01 M HCl and NaOH to pH 3 (H_3PO_4), 5.5 ($NaHCO_3$), 8 (NaH_2PO_4), 10 (Na_2CO_3), or 11.1 (Na_2CO_3). For ES/AS hydrolyses, the buffers (20 mL) were transferred to 20-mL amber glass vials (Fisher®, Pittsburgh, PA) and 50 μL of ES or AS in acetonitrile (ACN) was added to afford initial concentrations of 10 μM, below their estimated solubility of 0.2 mM at 25°C (*26*). Temperatures were maintained and mixing was controlled (170 rpm) with a Lab-Line® Environ-Shaker. Samples (1.0 mL), acquired as a function of time, were transferred to 4-mL amber glass vials pre-charged with 1.0 mL of hexane containing tetradecane internal standard at 0.8 μL/L hexane. Hexane-extractable analytes were removed from the buffer solutions by mixing for 2 min with a vortex Genie®. Emulsions were broken with ~100 mg NaCl and the hexane layer was analyzed with GC–MS. With a 25-μL syringe, duplicate 10 μL aliquots of the aqueous layer were removed, combined with 2 μL of ACN containing 6 μg of (+)-sclareolide (Sigma®, St. Louis, MO) external standard, and analyzed via HPLC–MS. For lactonizations, ES/AS HA aqueous stocks (pH 7) were added to 1 mL of 0.01 M buffer to afford initial concentrations of 0.9 mM in 2-mL glass vials; aqueous sampling was as described above.

Insects and oral secretions (OS)

Caribflies were cultured as described previously (*16*). Briefly, adult flies were separated by sex within two days after emergence from pupae. Sexes were kept in separate cages ($25 \times 25 \times 25$ cm^3) and rooms within a greenhouse. Each cage contained a water source and food, a 3:1 mixture by mass of table sugar to hydrolyzed yeast. After squeezing abdomens with fingertips until regurgitation, OS from 11–14-day-old sexually mature adults (*14*) were harvested with a glass capillary (1 mm i.d.) that penetrated a vial under slight negative pressure at 4°C. Collections were made 2 ± 0.5 h prior to sunset, pooled until ~0.6 mL was accumulated, and stored at −70°C.

Physicochemical characterization of oral secretions (OS)

An enzyme-coupled colorimetric assay was used to selectively determine concentrations of glucose, fructose, and sucrose in OS (*51*). A BEE-CAL™ microprobe was used to measure the pH of OS. Viscosity, μ (mPa · s), was measured using a Cannon–Manning Semi-Micro Viscometer (No. 75 and 350).

Pooled collections of OS (0.5 mL) were diluted with water to 1 mL. These samples were extracted with hexane and analyzed for ES/AS by GC–MS, or they were transferred to DSC-18 1-mL solid-phase extraction cartridges (Supelco®) that had been preconditioned and cleaned with sequential ACN (2×1 mL), methanol (2×1 mL), and water (3×1 mL) rinses. The cartridges were flushed with water (3×1 mL) to remove polar OS components such as salts and sugars. The analytes were then eluted into 4-mL tubes with rinses (3×1 mL) of 0.05% formic acid in 50% ACN. Eluants were concentrated via Speed Vac® to 0.5 mL and 120 μL of ACN containing 360 μg of external standard was added prior to HPLC–MS analysis.

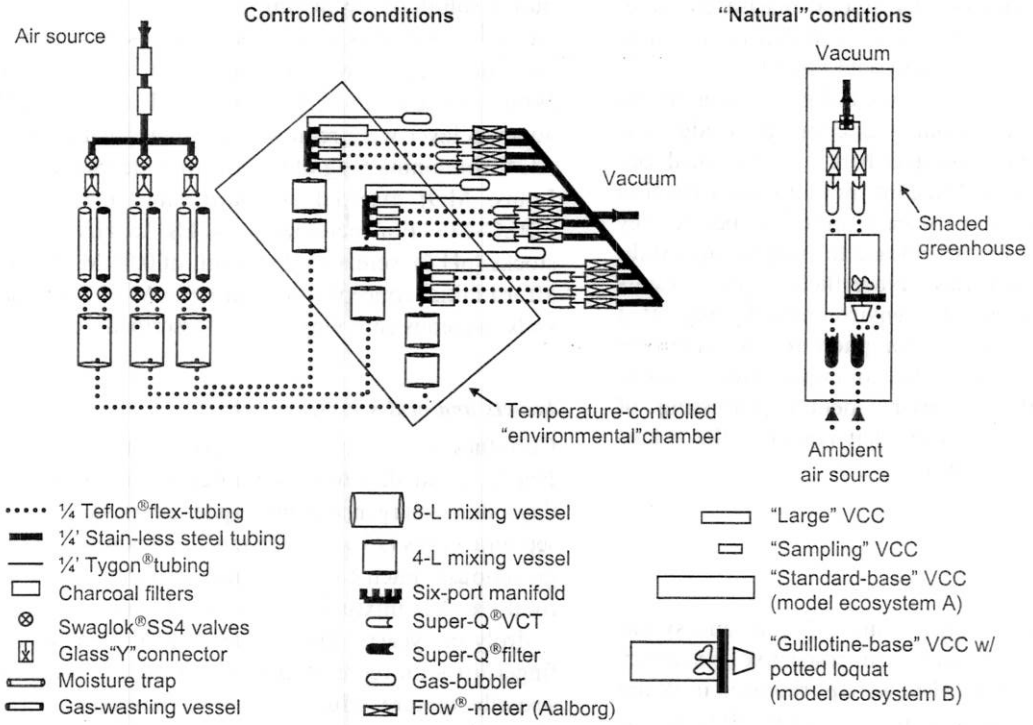

Figure 5. Schematic of volatile collection systems, modified from Heath and Manukian (*52*) that were used to determine the amount of airborne ES/AS pheromone, released from OS, under controlled and "natural" ambient atmospheric inputs of humidity and temperature.

Downloaded By: [University of California, Berkeley] At: 01:55 13 February 2010

Collection of volatile pheromones: fixed conditions

A modified volatile collection system (VCS) of Heath and Manukian (*52*) was used (Figure 5). A compressor pushed air (60 psi) through two Altech® L21 charcoal filters in series. Valves metered diversion into three streams that were subsequently split through a water-filled 500-mL gas washing bottle and a Drierite® filled moisture trap in parallel. The air streams of each pairing were metered to allow tunable absolute humidity (i.e. the concentration of water in air; $C_{a\text{-}H_2O}$) upon recombination in an 8-L glass-mixing vessel. The "conditioned" air supplies (three total) passed into two 4-L glass equilibration reservoirs in series and then a six-port manifold, all located within a temperature-controlled chamber. One port of each manifold was connected to a gas bubbler, located outside the chamber, which was used to verify a slight excess in airflow was maintained. Three Analytical Research Systems® (ARS) RV-A3 "sampling" volatile collection chambers (VCC) and a "large" 4CHB12R5 VCC, containing only a digital thermo–hygrometer, were connected to the remaining ports. Pheromones released from samples were captured on ARS glass tube (4 cm long × 4 mm i.d.) volatile collector traps (VCT) containing 20 mg Altech® Super-Q adsorbent. A 560 mmHg vacuum was metered to allow equivalent airflows (140–660 cm^3/min) between the VCCs.

Chamber temperature (20–40°C) and the absolute humidity of conditioned-air supplies, $C_{a\text{-}H_2O}$ (0.67–0.92 mM), were maintained for 6 h prior to substrate introduction. These conditions encompass those typical to caribflies' endemic range, the Greater Antilles and Florida (*45*). OS substrate was deposited (10 μL) onto 1"-square glass slides that were inserted into the "sampling" VCCs. Substrate consisted of either male OS, female OS, female OS supplemented with pheromone components, or 0.01 M $NaHCO_3$ buffer at pH 5.5 containing pheromone components and the same sugar concentrations present in OS (i.e. synthetic OS). VCTs were removed as a function of time and flushed with methyl *tert*-butyl ether (MTBE) (3 mL) into a volumetric glass vial pre-charged with 0.5 mL of MTBE containing tetradecane internal standard at 0.8 μL/L MTBE. The eluant was reduced to 0.5 mL with a gentle N_2 stream and analyzed by GC–MS. ES/AS collection efficiencies from synthetic OS were determined to be >98% over the range 5000–0.5 ng. Potential residual inputs of ES/AS from VCS components were below detection limits ($\sim 3.4 \times 10^{-9}$ M) in 30-day control

Downloaded By: [University of California, Berkeley] At: 01:55 13 February 2010

collections. After sampling was concluded, substrate humectant properties were examined; the diameter of the OS deposit was measured and, if possible, the volume was estimated by drawing it into a 25-μL syringe.

Collection of volatile pheromones: simulated natural conditions

Duplicate VCCs (ARS # 6CHB18R52) equipped with a "standard-base" (model ecosystem A) or a "guillotine-base" (model ecosystem B), which accommodated a young loquat tree (*Eriobotrya japonica*), were located within a shaded greenhouse. A vacuum was metered to provide airflow of 660 cm^3/min through each chamber as previously reported (*53*). Both VCC ends of each model ecosystem were fitted with VCTs; one functioned downwind to immobilize pheromones, the other (upwind) filtered a supply of ambient air from outside the greenhouse (Figure 5). After recording the temperature and humidity using a digital thermo–hygrometer housed within each model ecosystem, downwind VCTs were removed as a function of time, processed, and analyzed as described above.

For type A model ecosystems, VCC glass end/connecting fixtures were removed and replaced with nylon screen before 100 sexually mature male caribflies were introduced (with food and water sources (*16*)) and allowed to deposit OS "naturally" onto the glass from 10:00 PM to 9:00 AM the following morning. Prior to sampling ES/AS emitted from the OS, flies were removed and the fixtures were reincorporated. For type B model ecosystems, 10×10 μL of male OS was deposited onto either a 3" × 1" glass slide or the underside of loquat leaves at 9:00 AM.

Henry's law experiments

Dimensionless Henry's Law constants, equilibrium descriptors of air–water partitioning (*42*), are defined as:

$$K'_{\text{H-ES/AS}} = C_{a\text{-ES/AS}}/C_{\text{s-ES/AS HA}} \quad (7)$$

$$K'^{\text{sat}}_{\text{H-ES/AS}} = C_{a\text{-ES/AS}}/C^{\text{sat}}_{\text{s-ES/AS}} \quad (8)$$

and represent concentration (mol cm^{-3}) fractions of ES/AS in air ($C_{a\text{-ES/AS}}$) relative to ES/AS HA in solution ($C_{\text{s-ES/AS HA}}$) or ES/AS in solution under saturated conditions ($C^{\text{sat}}_{\text{s-ES/AS}}$). They were measured ($n = 8$) at 20, 25, 30, 35, and 40°C for 0.01M $NaHCO_3$ ($u = 0.1$M) at pH 5.5, female OS, and synthetic OS. The solution (1 mL) and an appropriate volume of pH 7 ES/AS HA aqueous stock (for $K'_{\text{H-ES/AS}}$) or ES/AS in ACN (for $K'^{\text{sat}}_{\text{H-ES/AS}}$), to afford concentrations of 0.9 mM, were encapsulated in a 2-mL septum-capped glass vial for 5 days. Prior to aqueous sampling (or hexane extraction) as described above, a needle fitted to a VCT was used to access vial headspace (~1 mL). Another needle, passing a N_2 stream at ~0.5 mL/min, was subsequently introduced to flush it. The VCT was removed after 10 min, processed, and analyzed as in the volatile pheromone collections.

Conclusion

Decades of research on using semiochemical attractants in pest management practices as "green" alternatives to broadcast insecticide application have outlined the importance of replicating the natural semiochemical release mechanism(s) utilized by a species and the marked influence that environmental factors can have on semiochemical release. In light of this knowledge and the example provided above, natural abiotic mechanisms for semiochemical release appear particularly well-suited for exploitation due to the intrinsic connectivity that exists between physicochemical-based inter-organism communication strategies and the abiotic environmental processing of the chemical signal. Specifically, this study points to the use of sugar-based solutions as media for the diffusion-controlled release of volatile insect aggregation pheromones. Unique features of this natural system, which are highly coveted when targeting the population control of flying insects, include release of volatile pheromones that is inversely related to temperature and directly related to relative humidity, as well as the ability to incorporate relatively hydrophilic pheromone precursors, linked to the volatile pheromone form through aqueous equilibria, into the media for the purpose of attenuating volatilization. Although the potential of sugar-based semiochemical release systems to serve sustainable agriculture is difficult to gauge presently, it is interesting to note the link between sugar-solutions and the ecology of many Diptera, Hemiptera, and Hymenoptera insect pests.

Acknowledgements

The United States Department of Agriculture financed this work. The colorimetric sugar assay was done by Dr. Sherry LeClere (USDA-ARS-CMAVE). We extend gratitude to James H. Tumlinson III (Pennsylvania State University and USDA-ARS-CMAVE) for his input on this research.

Electronic supplementary information

Additional methods, results, tables, and figures. This material is available free of charge via the online article page, from the multimedia tab.

Downloaded By: [University of California, Berkeley] At: 01:55 13 February 2010

References

(1) USGS. NAWQ Pesticide National Synthesis Project. http://water.usgs.gov/nawqa/pnsp (accessed Feb 14, 2009).

(2) USDA. APHIS. 2006. Exotic Fruit Fly Strategic Plan. http://www.aphis.usda.gov/plant_health/ea/downloads/ffeis.pdf (accessed Jan 6, 2009).

(3) Battiste, M.A.; Strekowski, L.; Vanderbilt, D.P.; Visnik, M.; King, R.; Nation, J.L. *Tetrahedron Lett.*. **1983**, *24* (26), 2611–2614.

(4) Chuman, T.; Sivinski, J.; Heath, R.R.; Calkins, C.O.; Tumlinson, J.H. *Tetrahedron Lett.*. **1988**, *29*(50), 6561–6564.

(5) Nation, J.L. *Environ. Entomol.*. **1975**, *4* (1), 27–30.

(6) Rocca, J.R.; Nation, J.L.; Strekowski, L.; Battiste, M.A. *J. Chem. Ecol.*. **1992**, *18*(2), 223–243.

(7) Butler, L.I.; McDonough, L.M. *J. Chem. Ecol.*. **1979**, *5*, 825–837.

(8) Heath, R.R.; Landolt, P.J.; Tumlinson, J.H.; Chambers, D.L.; Murphy, R.E.; Doolittle, R.E.; Dueben, B.D.; Sivinski, J.; Calkins, C.O. *J. Chem. Ecol.* **1991**, *17* (9), 1925–1940.

(9) Heath, R.R.; Teal, P.E.A.; Tumlinson, J.H.; Mengelkoch, L.J. *J. Chem. Ecol.* **1986**, *12* (12), 2133–2143.

(10) Nation, J.L. J. Chem. Ecol. **1990**, *16* (2), 553–572.

(11) Weatherston, I.; Miller, D.; Dohse, L. *J. Chem. Ecol.* **1985**, *11* (8), 953–978.

(12) Heath, R.R.; Epsky, N.D.; Landolt, P.J.; Sivinski, J. *Fla. Entomol.* **1993**, *76* (2), 233–244.

(13) Landolt, P.J.; Heath, R.R. *Pest Management in the Subtropics, Integrated Pest Management-A Florida Perspective*; Intercept Ltd: Andover, Hants, UK, 1996; pp 197–207.

(14) Nation, J.L. In *Fruit Flies their Biology, Natural Enemies, and Control;* Robinson, A.S.; Hooper, G., Eds.; Elsevier: Amsterdam, 1989; Vol. 3A; pp 189–205.

(15) Sivinski, J.; Epsky, N.D.; Heath, R.R. *J. Insect Behav.* **1994**, *7* (1), 43–51.

(16) Teal, P.E.A.; Lu, F. *Arch. Insect Biochem. Physiol.* **2001**, *48* (3), 144–154.

(17) Walse, S.S.; Lu, F.; Teal, P.E.A. J. *Nat. Prod.* **2008**, *71*, 1726–1731.

(18) Skoog, D.A.; West, D.M.; James Holler, F. *Fundamentals of Analytical Chemistry*, 7th ed.; 1996.

(19) Battiste, M.A.; Strekowski, L.; Coxon, J.M.; Wydra, R.L.; Harden, D.B. *Tetrahedron Lett.* **1991**, *32* (39), 5303–5304.

(20) Harris, D.C. *Quantitative Chemical Analysis*, 4th ed.; 1995, pp. 282–283.

(21) Baldwin, J.E. *J. Chem. Soc., Chem. Commun.* **1976**, *18*, 734–736.

(22) Perdue, E.M.; Wolfe, N.L. *Environ. Sci. Technol.* **1983**, *17*, 635–643.

(23) (a) Storm, D.R.; Koshland, D.E. *J. Am. Chem. Soc.* **1972**, *94* (16), 5805–5815; (b) Storm, D.R.; Koshland, D.E. *J. Am. Chem. Soc.* **1972**, *94* (16), 5815–5825.

(24) Strekowski, L.; Visnik, M.; Battiste, M.A. *Synthesis* **1983**, *6*, 493–494.

(25) Mabey, W.; Mill, T. *J. Phys. Chem. Ref. Data.* **1978**, *7*, 383–415.

(26) United States Environmental Protection Agency, EPI-Suite software [Online]. 2002. (a) WATERNT v1.00., (b) HENRYWIN v1.90., (c) MPVPWIN v1.41. http://www.epa.gov/opptintr/exposure/pubs/episuite.htm (accessed 14 Feb, 2009).

(27) (a) Prausnitz, J.M. *Molecular Thermodynamics of Fluid-Phase Equilibria;* Prentice Hall: Englewood Cliffs, NJ, 1969; (b) Przyjazny, A.; Janicki, W.; Chrzanowski, W.; Staszewki, R. *J. Chromatogr.* **1983**, *280*, 249–260.

(28) Covarrubias-Cervantes, M.; Champion, D.; Debeaufort, F.; Voilley, A. *J. Agric. Food Chem.* **2004**, *52*, 7064–7069.

(29) Kieckbusch, T.G.; King, C.J. *J. Agric. Food Chem.* **1979**, *27* (3), 504–507.

(30) Chandrasekaran, S.K.; King, C.J. *AIChE.* **1972**, *18* (3), 513–526.

(31) Friel, E.N.; Linforth, R.S.T.; Taylor, A.J. *Food Chemistry* **2000**, *71*, 309–317.

(32) Nawar, W.W. *J. Agric. Food Chem.* **1971**, *19* (6), 1057–1059.

(33) Lakshmi, T.S.; Nandi, P.K. *J. Phys. Chem.* **1976**, *80* (3), 249–252.

(34) Nahon, D.F.; Harrison, M.; Roozen, J.P. *J. Agric. Food Chem.* **2000**, *48*, 1278–1284.

(35) Fuller, E.N.; Schettler, P.D.; Giddings, J.C. *Ind. Eng. Chem.* **1966**, *58*, 19–27.

(36) Dey, P.C.; Motin, M.A.; Biswas, T.K.; Huque, E.M. *Monatshefte fur Chemie.* **2003**, *134*, 797–809.

(37) Contreras-Lopez, E.; Champion, D.; Hervert, H.; Blond, G.; Le Meste, M. *J. Agric. Food Chem.* **2000**, *48*, 1009–1015.

(38) Covarrubias-Cervantes, M.; Champion, D.; Debeaufort, F.; Voilley, A. *J. Agric. Food Chem.* **2005**, *53*, 6771–6776.

(39) Hikita, H.; Asai, S.; Azuma, Y. *CJChE.* **1978**, *56*, 371–374.

(40) Yamamoto, S.; Saeki, T.; Inoshita, T. *Chem. Eng. J.* **2002**, *86*, 179–184.

(41) Gilli, R. 1997. Momento on sugar. AvH Association. http://www.seas.upenn.edu/courses/belab/be309/SucroseCalculator.html.

(42) Schwarzenbach, R.P.; Gschwend, P.M.; Imboden, D.M. *Environmental Organic Chemistry;* John Wiley & Sons: New York, 1993.

(43) Carslaw, H.S.; Jaeger, J.C. *Conduction of Heat in Solids*; 2nd ed.; Oxford University Press: London, 1959.

(44) Crank, J. *The Mathematics of Diffusion*; Oxford University Press: London, 1975.

(45) SERCC. 2005. Historical Climate Summaries for Puerto Rico and the U.S. Virgin Islands. Columbia, SC. http://www.sercc.com/climateinfo/historical/historical.html (accessed Jan 6, 2009).

(46) Hughes, R.N.; Brimblecombe, P. *Agric. For. Meteorol.* **1994**, *67* (3, 4), 173–190.

(47) García-Legaz, F.M.; López-Gómez, E.; Beneyto, J.M.; Navarro, A.; Sánchez-Blanco, M.J. *J. Plant Phys.* **2008,** *165* (10), 1049–1060.
(48) Epsky, N.D.; Heath, R.R. *Environ. Entomol.* **1993,** *22* (2), 464–469.
(49) Burk, T. *Florida Entomol.* **1983,** *66* (3), 330–344.
(50) Hendrichs, J.P. PhD Thesis, University of Florida, Gainesville, 1986.
(51) (a) Hendrix, D.L. Crop Science ***1993,*** *33*, 1306–1311; (b) Tarpley, L.; Dahlberg, D.M.V.; Miller, FR. *Crop Science* **1993,** *33*, 338–341.
(52) Heath, R.R.; Manukian, A. *J. Chem. Ecol.* **1992,** *18* (7), 1209–1226.
(53) Heath, R.R.; Manukian, A. *J. Chem. Ecol.* **1994,** *20* (3), 503–604.

Downloaded By: [University of California, Berkeley] At: 01:55 13 February 2010