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The Development of Semiochemical Lures for Invasive Rats: An Integrated Chemical Image and Response-Guided Approach

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ABSTRACT: Olfactory lures are important tools in pest-species management, being widely used to monitor and trap populations. For vertebrates like rats, lures are most commonly foods such as peanut butter. However, these are perishable and require frequent replenishment; factors that decrease control operation efficacy and increase costs. Synthetic semiochemical-based lures might address these limitations, but their identification and use for vertebrate population management remains an underexploited opportunity. We used headspace solid-phase microextraction and gas chromatography-mass spectrometry to characterise the volatile chemical profiles of 19 food products previously presented to wild, free-ranging rats and assessed for attraction. Partial least squares regression identified 10 of the 111 compounds found in two or more analysed products to be statistically significant predictors of attraction. The identity of nine of the compounds was verified using authentic samples, while one was not commercially available. Field trials used tracking tunnels to present compounds at seven different concentrations from 10,000 ppm to 0.01 ppm. Inked cards inside tracking tunnels were used to quantify visitations using species tracking marks, with the presence of rat tracks on inked cards scored to provide a tracking rate. Five compounds outperformed peanut butter while eight individual semiochemical-based lures each statistically outperformed peanut butter. Nearly half of all confirmed visits to compounds were with lures presented at 0.1 and 0.01 ppm. A trend of increasing tracking rates with decreasing lure concentration was identified for aggregated compound data. Our results suggest a number of compounds have the potential for onward development as synthetic attractants for rats. Further, the results support our integrated chemical image and response-guided approach that statistically associated behavioural responses to a range of products with the volatile compounds in those products. This approach has the potential to identify semiochemical compounds, either allelochemical or pheromone, for use as olfactory lures for managing and monitoring a range of vertebrate pest species.

KEY WORDS: attraction, compound, gas chromatography-mass spectrometry, lure, olfactory, partial least squares regression, rat, *Rattus*, semiochemical, synthetic

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INTRODUCTION

Olfactory lures are important tools in wildlife management and conservation ecology, being widely used in monitoring and capture, reducing human-wildlife conflicts, influencing habitat movement, and eradicating pest species (Rosell and Kvinlaug 1998, Apfelbach et al. 2005, Kok et al. 2013). For invertebrates, the use of volatile semiochemicals as synthetic lures predominates and has been well exploited for decades (Witzgall et al. 2010). For example, methyl eugenol (a plant kairomone) has been used as an attractant to fruit flies (*Dacus* spp.) for nearly 100 years (Metcalf and Metcalf 1992). However, for vertebrates like rats, olfactory lures are commonly foods such as peanut butter. These items are, however, perishable and require frequent replenishment, factors that decrease control operation efficacy and increase costs (Parshad 2002, Astua et al. 2006, Murphy et al. 2014).

Synthetic semiochemical-based lures might overcome the limitations of food-based lures while offering additional benefits such as having temporally consistent odour properties, long life, ease of handling and storage, sex and/or behaviour-specific responses, and species specificity (Turkowski et al. 1979, Torto 2009, Shivik et al. 2014). However, to date, the identification and use of

semiochemical lures for vertebrate population management remains an underexploited opportunity. This may be because the identification of attractive semiochemical compounds, be they in urine, faeces, secretions, body odours, and foods, are likely to be a complex, challenging, and time-consuming task; factors that may have deterred researchers from beginning that search (Albone et al. 1986, Linklater et al. 2013).

The identification of semiochemicals commonly uses gas chromatography-mass spectrometry (GC-MS); a ubiquitous analytical technique in many fields such as forensics and medical research (Sparkman et al. 2011). However, datasets can often be large and complex. For example, McLean et al. (2012) report >150 compounds in Australian brushtail possum (*Trichosurus vulpecula*) cloacal samples. Identifying attractive semiochemical “compounds of interest” will therefore be challenging, as they will be found within datasets containing many hundreds of compounds, most of which have no influence on animal behaviour.

Commonly used methods to interrogate GC-MS datasets, such as selecting compounds based on their presence and relative abundance, also present problems for identifying semiochemicals. This is because the

amount of a compound is not predictive of its influence. Many compounds found at low concentrations are important influencers while other, more abundant compounds are not. Instead, a better approach is to link the semiochemicals found in products to behavioural responses to those products, i.e., an integrated chemical image and response-guided approach (Albone et al. 1986).

The aim of our research was, therefore, to identify attractive semiochemical compounds in food products that could form the basis of a synthetic lure for rats, using a combined chemical image and response-guided approach. In this paper, we detail the use of headspace solid phase microextraction (HS-SPME) coupled with GC-MS to identify the volatile profiles of 19 food products and a control presented to wild, free-ranging rats. We demonstrate the use of partial least squares regression (PLSR) to statistically associate compounds to a behavioural response with the aim of identifying those compounds likely to elicit attraction. We then summarise the results of our bioassays using nine statistically significant compounds identified by the PLSR analysis. To the best of our knowledge, this is the first time GC-MS data outputs and PLSR have been combined in such a way to identify semiochemicals, either allelochemical or potentially pheromone, with the aim of applying those findings to the development of a synthetic olfactory lure.

METHODS AND MATERIALS

Twenty products (19 foods and one control apparatus) from our previous research (Jackson et al. 2015) were subjected to HS-SPME GC-MS analysis to generate a list of volatile chemical compounds found in each product. Samples were prepared 24 hours prior to analysis, with five g of sample added to each of three replicate headspace screw vials and sealed using a PTFE/silicone septa screw cap (Agilent Technologies, Santa Clara, CA, USA). Sampling was undertaken using a manual SPME injection apparatus fitted with a pre-conditioned PDMS/Carboxen/DVB SPME fibre (Supelco, Sigma-Aldrich, St. Louis, MO, USA). Each sample vial was incubated in a water bath for 15 minutes with the SPME fibre then injected into the GC-MS injector port and thermally desorbed for two minutes. Analysis used a Shimadzu QP2010 Plus Gas Chromatograph-Mass Spectrometer fitted with an Rxi[®]-5-Sil MS capillary column and a Restek[®] SPME liner. Compounds were identified by comparison of EI fragmentation mass spectra with those contained in the NIST11 library. Those with a similarity score of >85% in at least two of three replicates were carried forward for statistical analysis. Statistically significant compounds as identified by PLSR were validated with retention indices generated by the retention times of a series of n-alkane standards (C₈-C₄₀; Sigma-Aldrich). Validated compounds were purchased from AK-Scientific (Union City, CA, USA) and Sigma Aldrich, and their identity verified by comparison of both EI fragmentation pattern and retention time.

Data Analysis

Data were subjected to partial least squares regression using Unscrambler[®] X 10.3 (CAMO, Oslo, Norway). Analysis used the Nonlinear Iterative Partial Least Squares (NIPALS) algorithm and was validated using random cross-validation. Martens' Uncertainty Test was used to identify the chemical compounds in analysed products that were statistical predictors of attraction. The largest peak area for each compound was used as the predictor variable while the attractiveness score for each product (Jackson et al. 2015 supplementary information) was used as our response variable. All X variables were log transformed. All X and Y variables were mean centred and scaled to one standard deviation. Those compounds found only in one food product were removed from analysis as they provided no information (Wold et al. 2001).

Field Trials

Synthetic lures comprising a single compound mixed in a carrier medium were presented to wild, free-ranging rats at seven concentrations, decreasing in orders of magnitude from 10 from 10,000 ppm to 0.01 ppm. Lures were presented in 1.7 mL Eppendorf microtubes secured to the inside wall of tracking tunnels using a cable tie. Inked cards were placed in each tunnel to quantify the visitation and identity of species visiting lures. Lures were randomly assigned along transects, with a minimum 25-m spacing between lures and with transects separated by 200 m. A control (carrier medium only) and standard (peanut butter) were assigned to each transect, with each transect comprising of lures presented at the same concentration. The order of transects was randomised for each trial. All lures were left *in situ* for one rain-free night. Lures were scored using the presence of rat tracks on inked cards to provide a proportion of inked cards receiving a visit for each lure, hereafter termed the "tracking rate." Binomial tests were used to compare the tracking rate of each individual lure against peanut butter at the end of the trial period. Binomial tests were run in R (R Core Team 2015). Ten trials were undertaken at independent sites across the Wainuiomata and Orongorongo catchment region and within the Akatarawa and Pakuratahi Forest Parks, Wellington region (41°15S, 175°00E), New Zealand between 15 July 2015 and 11 November 2015.

RESULTS

GC-MS analysis characterised 375 compounds across the 20 sampled products, 111 of which were found in two or more of the analysed products and carried forward to PLSR analysis. Partial least squares regression identified 63 compounds that were positively correlated to the response variable, with ten compounds identified as statistically significant predictors. Statistically significant compounds were derived using the first two factors of the PLSR model that together explained 91% of the validated variance in the dataset (Figure 1). Model cross-validation

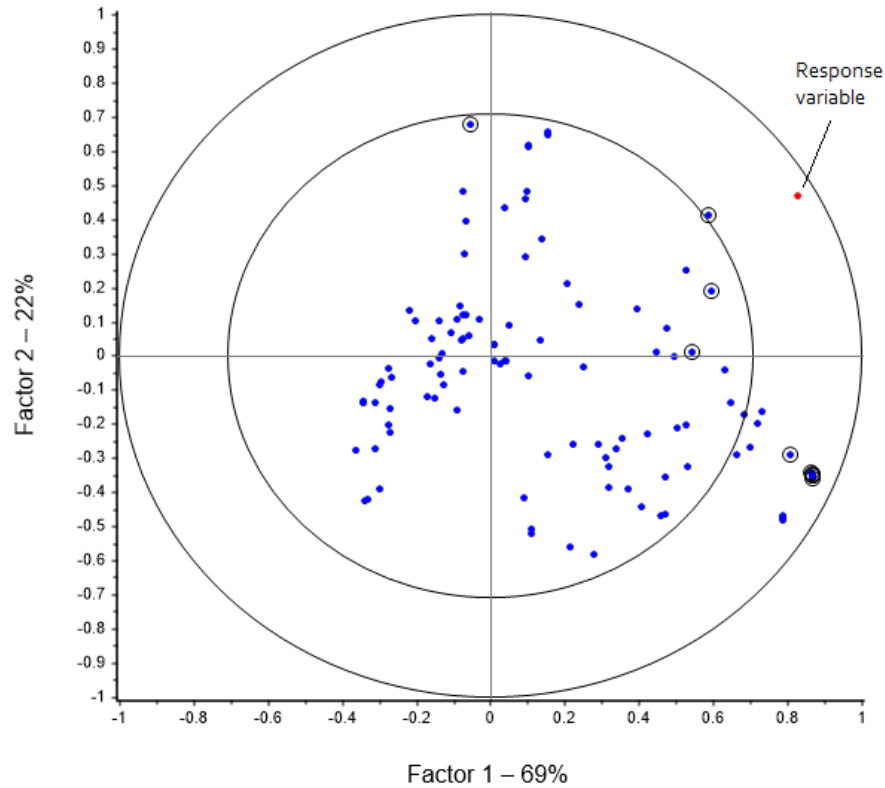


Figure 1. Partial least squares regression plot of the models first two factors and associated explained variance. Points are compounds while circled points are compounds statistically significant to the model. The response variable is indicated. Negative markers on the x and y axis indicate negative correlations to that factor while positive markers indicate positive correlations to factors.

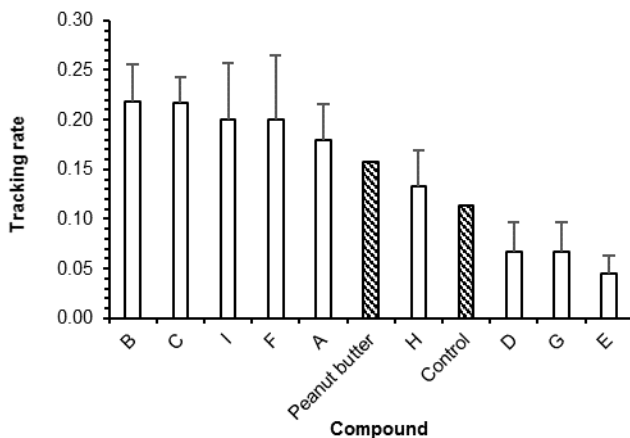


Figure 2. Tracking rate for each compound (letter codes), control, and standard. The tracking rate for each compound is presented as the aggregated tracking rate for all seven concentrations of that compound. The peanut butter standard and the control are shown hatched to provide visual differentiation. Compound tracking rates provided $\pm 1SE$.

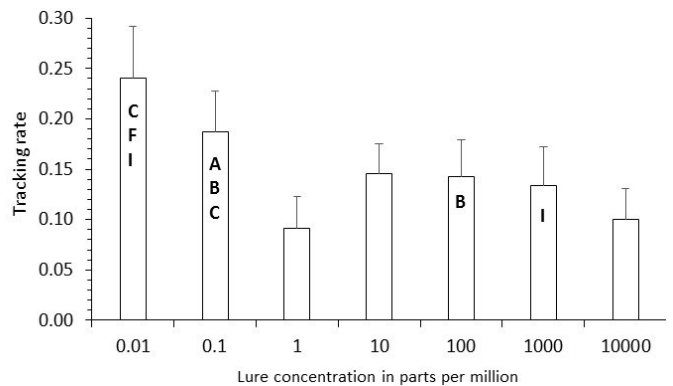


Figure 3. Tracking rates for all compounds aggregated based on concentration. Letter codes in columns denote the eight statistically significant individual lures while the column they are in indicates the concentration at which they were significant. Tracking rates are provided $\pm 1SE$. The combined compound data show an increasing trend in tracking rates with decreasing concentration.

for the two factors was $r^2 = 0.54$. Of the ten statistically significant compounds, the identities of nine were positively verified using authentic standards and carried forward to field trials. The nine compounds were provided letter codes from A to I. The remaining compound was not commercially available.

Field Trials

Five compounds (B, C, I, F, and A) were more attractive than the peanut butter standard, one compound (H) was less attractive than the peanut butter standard but more attractive than the control, and three compounds (D, G, and E) were less attractive than the peanut butter standard and the control (Figure 2). Eight individual lures statistically outperformed the peanut butter standard ($p < 0.01$), with all eight coming from the five top-performing compounds (Figure 3). Attraction to compounds was highest at the two lowest concentrations, with nearly half of all recorded visits occurring with lures presented at 0.1 ppm and 0.01 ppm (tracking rates 0.19 and 0.24, respectively). The lowest recorded tracking rate (0.09) was for lures presented at 1 ppm (Figure 3). With the exception of compound G (no trend), attraction to individual compounds showed an increasing trend with decreasing concentration.

DISCUSSION

Our research identified a number of compounds that were attractive to rats, with five compounds outperforming peanut butter as a lure. This is interesting, as mammalian olfaction is often suggested to be tuned to identify and interpret complex mixtures, not individual compounds (Albone et al. 1986). For example, although olfactory receptors have odour specificity, it is suggested pheromones gain their species-specificity from a combination of molecules that only work as a whole (Wyatt 2014). Our research, however, indicates that rats identify and interpret single compounds. This suggests the development of semiochemical lures for rats may not require the complex multicomponent blends previously suggested. That said, this does not preclude multi-component blends from offering higher levels of attraction if the right combination of compounds could be identified. Indeed, we are currently presenting the top five compounds (B, C, I, F, and A) at their optimal concentrations as dyad, triad, and tetrad multicomponent blends to test for synergistic relationships that may increase the attraction of these synthetic lures.

We identified higher tracking rates at low concentrations and an increasing trend in attraction with decreasing concentration. In fact, six of the eight statistically significant lures were from the two lowest concentrations. Given the dominance of olfaction to rats and most vertebrates (Solomon et al. 2007), we would therefore recommend that bioassays assessing the attractiveness of semiochemicals on vertebrates should consider presenting compounds at those lower concentrations. That said, the physical properties of individual compounds, such as vapour pressure, and the animal's detection threshold for that compound, may demand that a broad spectrum of concentrations are at least considered. Nonetheless, our findings suggest a bioassay that initially focuses on lower

concentrations may prove a more fruitful approach, with higher concentrations trialled if attraction rates at low concentrations are poor. Furthermore, given the increasing trend in tracking rates for all compounds with decreasing concentration, consideration should be given to presenting compounds at concentrations lower than those presented by us, for example, at parts per trillion.

In conclusion, our research identified eight individual lures from five compounds that were attractive to rats, and these have formed that basis of our ongoing development of synthetic lures. These synthetic lures will help overcome many of the limitations of food-based lures and, when married with existing semiochemical dispensing technologies, will have the additional benefit of ease of handling, consistent odour delivery, and longevity. Furthermore, future testing will allow us to consider the species specificity of these lures, a highly desirable commodity for reducing non-target impacts (Campbell et al. 2015). Lastly, we effectively demonstrated an integrated chemical image and response-guided approach by statistically associating behavioural responses to products with the volatile compounds found in those products. This approach has the potential to identify semiochemical compounds, either allelochemical or pheromone, for use as olfactory lures for managing and monitoring a range of vertebrate pest species.

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