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CHEMICAL SIGNALING IN ASIAN ELEPHANTS (*ELEPHAS MAXIMUS*): CONCENTRATION EFFECTS WITH APPLICATIONS FOR MANAGEMENT AND CONSERVATION

A Thesis
Presented to
The Faculty of the Department of Biology
Western Kentucky University
Bowling Green, Kentucky

In Partial Fulfillment
Of the Requirements for the Degree
Master of Science

By
Chase Andrew LaDue

August 2016

CHEMICAL SIGNALING IN ASIAN ELEPHANTS (*ELEPHAS MAXIMUS*): CONCENTRATION EFFECTS WITH APPLICATIONS FOR MANAGEMENT AND CONSERVATION

Date Recommended 8 July 2016

Bruce A. Schulte, Ph.D., Director of Thesis

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Michael E. Smith, Ph.D.

Dean, Graduate School 7/11/16 Date

This work is dedicated to the memory of Dr. L.E.L. 'Bets' Rasmussen, who I never had the fortune of knowing, but whose enduring commitment, creativity, and enthusiasm for elephants I continually strive to follow.

"I firmly believe in my own research. If I didn't believe in it, I would have quit about five years ago…That's one thing you learn about working with elephants—you learn patience."

-Bets Rasmussen

(from Tisdale, 1989)

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I am humbled to have the support of the elephant community for this work, as is evident by the Association of Zoos and Aquariums' Elephant Taxon Advisory Group's

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CHEMICAL SIGNALING IN ASIAN ELEPHANTS (*ELEPHAS MAXIMUS*): CONCENTRATION EFFECTS WITH APPLICATIONS FOR MANAGEMENT AND CONSERVATION

Chase A. LaDue August 2016 131 Pages

Directed by: Dr. Bruce A. Schulte, Dr. Jarrett R. Johnson, and Dr. Michael E. Smith

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Asian elephants utilize two chemical signals that have been described to function in reproduction: (1) (*Z*)-7-dodecenyl acetate (*Z*7-12:Ac) is released by females near ovulation, and (2) frontalin is released by males around the time of musth. Signaling theory posits that the concentration at which either compound is emitted should have implications for the response of the receiver, varying with factors such as sex and reproductive experience. Here, the objectives were to: (1) investigate the effect of concentration on receiver chemosensory behavior in an effort to identify detection thresholds and concentrations of maximum response for reproductively experienced or inexperienced male and female Asian elephants, and (2) characterize the broader behavioral impacts of each of these compounds in an effort for application as environmental enrichment in captive settings. Concentrations from 0.0 mM to 2.0 mM of both frontalin and *Z*7-12:Ac were bioassayed simultaneously with captive elephants housed at facilities across North America in two experiments: one that tested mid-range concentrations and a second that tested low and high concentrations. There was a general increase in chemosensory response with increasing concentration of both compounds regardless of sex or reproductive experience. Females exhibited a lower detection threshold for frontalin, and the opposite was true for males with *Z*7-12:Ac. Reproductive experience also influenced thresholds: inexperienced males had a higher threshold than

experienced males for frontalin (the same was true for females), and experienced males were able to detect *Z*7-12:Ac samples as low as 10^{-7} mM. Aside from inexperienced males, all elephants responded maximally to the 1.0 mM samples of both compounds. Elephants exposed to mid-range concentrations of either compound showed no notable changes in behavior after application of the signals, although inexperienced males spent less time inactive and more time walking after frontalin bioassays, and inexperienced females foraged more after exposure to *Z*7-12:Ac. Interpreted together, this suggests that the concentration at which either compound is emitted has strong implications for chemosensory response based on the identity of the receiver in Asian elephants, although it is unclear whether these compounds have other behavioral effects that can be targeted for a goal-oriented olfactory enrichment program.

CHAPTER 1

INTRODUCTION TO THESIS

One critical property of a chemical signal that can affect how it is perceived by its receiver is concentration (e.g. Foster & Johnson, 2011; Harris, Keller, & Miller, 1987; Linn, Bjostad, Du, & Roelofs, 1984; Lönnstedt & McCormick, 2011). I sought to better characterize how Asian elephants (*Elephas maximus*) respond to chemical signals of various concentrations. This thesis is organized into two main sections: Chapter 2 describes the behavioral response patterns of Asian elephants to increasing concentrations of two chemical signals in the light of signaling theory, while Chapter 3 investigates the broader effects of both of these compounds on the behavior of captive elephants with direct implications for enhancing olfactory enrichment programs. For this thesis, it would have been inefficient (and impractical) to conduct a detailed study on the effects of chemical signal concentration on Asian elephants in the wild. As such, the studies described here were conducted on captive Asian elephants in readily accessible facilities across North America. In doing these captive studies, it is possible to manipulate the environment to provide better conditions for signal reception, and the complete life histories of each elephant (including sex, age, relatedness, and reproductive status/history) are generally known. Therefore, I was able to sample a wide range of elephants of varying sexes, ages, and reproductive experiences, making it possible to better understand how the condition of a receiver affects signal perception at various concentrations.

Animals rely on signals to communicate qualities that may not be directly perceivable, where signals are defined as acts or structures that purposely alter the

behavior of others (Searcy & Nowicki, 2005; Slater, 1977). Implicit in this definition is that the signal evolved for a particular purpose, and the receiver of the signal has evolved a concomitant response (Maynard Smith & Harper, 2003). Signal theory posits that animals use signals that represent trade-offs between production and release costs, and benefits accrued by accurate reception. Signals occur through a variety of sensory modalities, but perhaps the most pervasive method utilized across all taxa is chemical communication (Wyatt, 2014). Through the scope of signal theory, chemical communication may be advantageous over other channels because chemical signals are often linked to physiological processes that are difficult to fake; that is, they are honest indicators of a sender's condition (Maynard Smith & Price, 1973). Additionally, chemical signals can often last longer than signals conveyed through other sensory modalities (e.g. visual, auditory, tactile). Therefore, it is of great interest to study chemical signals in the light of signal theory because these signals possess unique qualities and are shared by all organisms.

Pheromones are intraspecific chemical signals released by one individual (the sender) to induce a behavioral and/or physiological change in a conspecific (Karlson & Lüscher, 1959). Despite the diversity of pheromones described, recognition of pheromone functionality is still growing: over 3,500 chemical signals have been reported in animals, but exceedingly few (approximately 31) are from vertebrates (Dulac & Torello, 2003; Tirindelli, Dibattista, Pifferi, & Menini, 2009). Of these vertebrate pheromones, 16 are complex mixtures with a variety of molecular structures and functions (Apps, 2013; Apps, Weldon, & Kramer, 2015). Mammals in particular secrete pheromones through their breath, urine, feces, saliva, and glands, and they generally

detect these signals via the main olfactory and vomeronasal systems (Albone, 1984; Evans, 2003). Chemical signaling has been shown to serve a variety of functions—including social organization, reproductive synchrony, and predator recognition—that mirror the utility of these signals in invertebrate species (Wyatt, 2005). Despite this, the complexity of mammalian "signature mixtures" reported by many may discourage further pursuit towards the discovery and identification of signals in mammals and other vertebrates (Wyatt, 2010). Apps (2013) suggests that these expectations may be unfounded; there may be single-component pheromones, simple mixtures, and/or other qualitative differences between signals that are simply overlooked in the pursuit to better understand vertebrate chemical signaling.

This thesis builds upon the pioneering work of L.E.L. Rasmussen and colleagues, who were the first to isolate and identify two single-compound pheromones in Asian elephants (*E. maximus*). (*Z*)-7-dodecenyl acetate (*Z*7-12:Ac) is a pheromone released in the urine of females during the preovulatory phase of estrus (Rasmussen, Lee, Zhang, Roelofs, & Daves, 1997). Males presumably use *Z*7-12:Ac to determine when females are receptive to breeding (Rasmussen & Greenwood, 2005; Rasmussen, Krishnamurthy, & Sukumar, 2005). Musth is a regularly occurring, heightened reproductive period during which male elephants undergo an array of behavioral and physiological changes that are triggered by a surge in plasma testosterone (Jainudeen, Katongole, & Short, 1972). Males compete with each other for access to females, and so intersexual signals that mediate the synchronization of breeding would theoretically benefit both sender and receiver. In turn, male elephants in musth emit another pheromone called frontalin; Rasmussen and Greenwood (2003) hypothesized that frontalin serves to signal

reproductive intent to females and diffuse competition between males. There is evidence to support similar inter- and intrasexual pheromones occurring in African elephants (*Loxodonta africana*), but single compounds and multi-compound mixtures have not been functionally identified (Bagley, Goodwin, Rasmussen, & Schulte, 2006; Castelda, Goodwin, & Schulte, 2008; Meyer, Goodwin, & Schulte, 2008). Pheromones appear to play a large role in the social organization of Asian elephants, but as with most vertebrates, our understanding of chemical signaling and reception in *E. maximus* remains limited.

The purpose of this study was to characterize how Asian elephants respond to different concentrations of biologically relevant chemical signals: *Z*7-12:Ac (the female estrous pheromone) and frontalin (the male musth pheromone). Chapter 2 describes the response patterns of elephants towards various concentrations of each of these signals, and Chapter 3 investigates the efficacy of applying these signals for captive elephant management. Together, this provides invaluable information for *in-situ* human-elephant conflict mitigation strategies, with potential to enhance the daily husbandry and long-term management of *ex-situ* populations—these applications are discussed in more detail in Chapter 4. The biological relevance of these signals is important, because their meaning is continuously reinforced through natural interactions with conspecifics; therefore, these signals may have a high potential for success in any management applications for wild or captive elephant populations.

CHAPTER 2

THE BEHAVIORAL EFFECTS OF CHEMICAL SIGNAL CONCENTRATION IN ASIAN ELEPHANTS

Introduction

Signaling theory (also referred to as signal detection theory) predicts the instances in which animals should send and respond to signals, defined as acts or structures that purposely alter the behavior of others (Reeve, 1989; Searcy & Nowicki, 2005; Slater, 1977). There are obvious scenarios in which responding to a signal is beneficial to the sender and/or receiver, but perhaps less obvious are the inherent costs associated with honest signaling. Responses have evolved together with their corresponding signals, so they are also subject to selective pressure; according to optimality theory any observed responses should be a result of a trade-off between costs and benefits (Houston, Clark, McNamara, & Mangel, 1988; Mangel & Clark, 1988).

Signals should evolve to be reliable so that the costs of incorrectly responding to dishonest signals are minimized. The mechanisms that protect the reliability of signals are still discussed, but the handicap principle first described by Zahavi (1975)—suggesting that signals have some measurable cost to the signaler and are therefore inherently honest—has been among the most widely accepted. These signaling costs have been distinguished between the costs needed to produce and transmit the signal (i.e. efficacy costs) and the costs associated with wasteful signals (i.e. strategic costs). However, Számadó (2011a,b) reviewed instances when the handicap principle did not accurately predict signal honesty. In these cases, higher quality signalers do not necessarily incur higher costs, weaker signalers often employed signals despite high

costs, or honest signals did not necessarily handicap their signalers (e.g. Bergstrom & Lachmann, 1998; Getty, 2006; Hurd, 1997). Chemical signaling is unique compared to other sensory modalities: chemical signals are considered difficult to fake because they are directly linked to physiological processes (i.e. they are inherently 'honest' signals of condition; Maynard Smith & Price, 1973). In light of alternative theories to the handicap principle, optimal conditions under which to produce and respond to chemical signals may differ from those of other sensory modalities.

Signals of the same chemical structure have been shown to vary in meaning based on the concentration at which they are emitted in invertebrates and vertebrates (e.g. Blum, 1996; Coureaud, Langlois, Sicard, & Schaal, 2010; Srinivasan et al., 2008; Wyatt, 2014). This phenomenon—termed pheromonal parsimony—refers to signals of the same chemical composition that change meaning based on contextual variables (concentration, social access, etc.). For example, male moths (*Heliothis virescens*) respond only to high concentrations of the female sex pheromone—only unstressed females are capable of producing the pheromone in high concentrations, so the male response is thought to be adaptive (Foster & Johnson, 2011). Similarly, bark beetles (*Ips pini*) exposed to increasing concentrations of their aggregation pheromone exhibit diminished responses at exceedingly high concentrations (Erbilgin, Powell, & Raffa, 2003). The concentration at which signals are emitted can also affect the meaning of the message entirely. In termites (e.g. *Pseudacanthotermes spiniger*), males perceive signals emitted by females at low concentration as trail pheromones but at high concentrations as sex pheromones (Bordereau & Pasteels, 2011). Besides concentration and other chemical properties, the same pheromone may have different meanings depending on the social and

environmental context in which it is presented. For example, ants (*Temnothorax rugatulus*) in an unfamiliar nest release 2,5-dimethylpyrazine as a signal to conspecifics to reject the nest as their own; the same pheromone is used as an aggregation pheromone when released outside an ant's home nest (Sasaki, Hölldobler, Millar, & Pratt, 2014). In any situation, the chemical properties (e.g. concentration) and context in which a signal is received should be considered in determining overall function. Specifically, there is often biological relevance to high or low concentrations of chemical signals, and concentration may therefore serve to modulate the response of the receiver towards a signal. Thus, these contextual factors may be relevant for reproductive signaling for vertebrates as well as invertebrates. While relatively few pheromones have been identified for vertebrates (Apps et al., 2015; Tirindelli et al., 2009), two are known to be related to reproductive behavior in the Asian elephant (*Elephas maximus*), thereby driving the motivation for this study.

The elephant estrous cycle is 14 to 16 weeks long, with a 4- to 6-wk follicular phase and an 8- to 12-wk luteal phase (Plotka et al., 1988). Elephants show two luteinizing hormone (LH) surges during their estrous cycles: the first LH surge occurs approximately 3 wks before ovulation to cause nonovulatory follicles to form accessory corpora lutea, and the second LH surge occurs around ovulation (Brown, 2000). *Z*7-12:Ac is first detectable in female Asian elephant urine after the luteal phase, and it increases in concentration linearly with the progression of the follicular phase through ovulation (Rasmussen, Lee, et al., 1997; Rasmussen, 2001). This pattern is hypothesized to aid in the synchrony of reproduction between sexes (Rasmussen et al., 2005).

In a similar fashion, male Asian elephants in musth exude frontalin via temporal gland secretions, urine, and breath, and the concentration of frontalin changes within a single musth episode and increases in concentration during musth as a male ages (Rasmussen & Greenwood, 2003). Additionally, male Asian elephant temporal gland secretions contain both enantiomers of frontalin, (+) [1R,5S] and (–) [1S,5R]; (Greenwood, Comeskey, Hunt, & Rasmussen, 2005). Moda musth is a period experienced by young males that is characterized by erratic behavior and a range of sweet-smelling temporal-gland secretions; it is thought to function by diverting unwanted attention from older males. Additionally, young moda males have a higher proportion of the (+) frontalin enantiomer, but older males secrete (+) and (–) enantiomers in equal proportion. Moda males generally avoid frontalin samples with equal enantiomeric ratios, but samples resembling moda secretions elicit little response from mature males (Rasmussen, Riddle, & Krishnamurthy, 2002). Similarly, female Asian elephants in the follicular phase of estrus respond differently to the enantiomeric ratio and concentration of frontalin (Rasmussen, Greenwood, Goodwin, & Schulte, 2016). There is also evidence that elephants respond according to their own physiological status: Rasmussen and Greenwood (2003) reported that pregnant females are cautious when exposed to frontalin, yet preovulatory females are attracted to the signal and luteal females appear indifferent. Older males do not appear to be affected by frontalin, but younger males are repelled when exposed to older males secreting frontalin or from the chemical signal itself (Rasmussen et al., 2002). Similarly, *Z*7-12:Ac may be irrelevant to female conspecifics, while males are expected to be attracted to it. Both *Z*7-12:Ac and frontalin

appear to exhibit concentration-dependent effects, and these effects may be compounded by the context in which they appear (e.g. the status of the receiver).

The detection threshold of a chemical signal may vary based on the condition of the receiver (Roelofs, 1978). Optimality theory predicts that animals should only react to signals above a certain concentration, and that it is indeed costly to continuously respond to a signal below that concentration. Numerous other contextual factors may influence the detection threshold of a signal. For example, flowers of *Tripterygium hypoglaucum*, the principal nectar source of honeybees (*Apis cerana*), emit a chemical called triptolide. Bees adjust their triptolide detection threshold based on the availability of nectar; when resources are scarce, this threshold lowers (Tan et al., 2007). Similarly, stingless bees (*Melipona asilvai*) adjust the detection threshold for nestmate recognition based on the similarity of a non-nestmate's cuticular hydrocarbon profile (Nascimento & Nascimento, 2012). The detection threshold of many signals used by mammals may be exceedingly low. Even mammals that do not appear to rely on olfaction as a primary sense, such as the squirrel monkey (*Saimiri sciureus*), have a high olfactory sensitivity (down to 1.6×10^{-8} mM for a neutral odorant, amyl acetate; Laska, Seibt, & Weber, 2000). Investigation of how detection thresholds change may elucidate the relative importance of various signals to receivers in an array of physical and physiological conditions.

Descriptions of chemosensory response patterns along a signal's concentration gradient are generally lacking in the literature, but research that investigates how animals respond to varying concentrations of a signal—and how the condition of the receiver affects signal perception—is certainly warranted. The relationship between response concentration can follow several patterns with increasing signal concentration.

Individuals may show more frequent or intense behavioral responses as the concentration of chemosignal increases in a linear fashion (e.g. Mirza & Chivers, 2003). A similar pattern may occur when individuals exhibit greater responses until a certain chemical concentration is reached, and then the response remains at a constant rate in a logistic increase pattern (e.g. Beyers & Farmer, 2001; Laska et al., 2000). Hypothetically, opposite results are possible with patterns of linear decrease (responses are highest at or near the detection threshold) or logistic decay. Another possibility is that responses follow the pattern of a bell curve, whereby responses are greatest at an intermediate range of concentrations.

Studies on captive Asian elephants (Arvidsson, Amundin, & Laska, 2012; Rizvanovic, Amundin, & Laska, 2013) have shown that individuals are able to discriminate between structurally similar odors at rates at least as well as mice (Bodyak & Slotnick, 1999), rats (Slotnick, Kufera, & Silberberg, 1991), and dogs (Lubow, Kahn, & Frommer, 1973), and clearly better than primates, including humans (Laska & Teubner, 1999). However, the chemical signals used in these discrimination trials were of little biological significance to elephants, and detection thresholds for biologically relevant signals (e.g. pheromones) may be substantially lower. While detailed observations of investigatory behavior towards suspected biologically relevant signals in Asian and African elephants have been reported (e.g. Rasmussen & Schulte, 1998; Schulte et al., 2005; Schulte et al., 2013), systematic evaluations of threshold levels and concentration effects of *Z*7-12:Ac and frontalin have not been completed. Rasmussen, Lee, et al. (1997) presented varying concentrations of *Z*7-12:Ac to a limited sample size

of nine captive male Asian elephants, showing that there may be intriguing concentration effects involved with this signal.

The objectives for this study were to better characterize the response patterns of reproductively experienced and inexperienced male and female Asian elephants of to changing frontalin and *Z*7-12:Ac concentrations. I hypothesized that the concentration of either signal would influence the receiver's behavior, and this response would vary with both sex and reproductive experience. Based on the sex and reproductive experience of the receiver, I also attempted to identify detection thresholds and concentrations of maximum response for each compound.

Methods

Study sites and subjects

According to the latest records from the Association of Zoos and Aquariums' Asian Elephant Species Survival Plan® (Keele, 2015), there are 254 Asian elephants (55 males and 199 females) held at 55 institutions (e.g. zoos, wild animal parks, circuses, private owners) in North America. Bioassays were conducted at 14 (25%) of these facilities. The sample population consisted of 73 elephants: 28 males (ranging from 1 – 53 years old, median age = 17 y) and 45 females (ranging from 6 mo – 64 y, median age = 36 y). Details on each elephant—including age, sex, reproductive status (if known), and social access during the bioassays—are provided in Appendix I.

Description of experiments

This chapter describes two separate experiments: the first experiment assayed mid-range concentrations of frontalin and *Z*7-12:Ac, while the second experiment tested higher and lower concentrations for each compound (along with a single mid-range concentration and a buffer control for comparison to the first experiment). Originally I expected the ranges assayed during the first experiment would indicate the detection threshold and concentration of maximum response for each compound. When this was not the case, the second experiment was designed to identify these values. The concentrations assayed in the first experiment were chosen to mirror concentrations similar to the ranges observed in adult elephants (Rasmussen, 2001; Rasmussen & Greenwood, 2003). However, the maximum and minimum concentrations assayed in the second experiment are not beyond the ranges described to occur naturally.

The chemical samples presented for each bioassay during the first experiment were 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 0.0 mM samples of each compound, along with a 1.0 mM vanillin sample. The 0.0 mM and vanillin samples were used as controls. Vanillin is found in small amounts in Asian elephant urine, and elephants have been shown to exhibit low rates of chemosensory behavior towards vanillin samples (Schulte & Rasmussen, 1999). The samples for each compound presented during the bioassays in the second experiment were 2.0, 1.0, 10^{-2}, 10^{-5}, 10^{-7}, and 0.0 mM (a 10^{-2} mM sample was presented to connect the results of both experiments). Vanillin samples were not presented during the second experiment; the 0.0 mM sample was used as the sole control.

The sample size for the first experiment was considerably larger, and included 52 elephants from 10 facilities. The second experiment included 29 elephants from 6

facilities. Eight elephants (from 2 facilities) were assayed in both experiments (Appendix I).

Chemical sample preparation

Chemical preparation procedures followed protocols established by Rasmussen (2001), Rasmussen and Greenwood (2003), and Rasmussen et al. (2003). Chemical samples were prepared less than 24 hours before each bioassay. Each compound was dissolved in 10^{-2} M sodium phosphate buffer diluted from 10^{-1} M sodium phosphate buffer, as follows: 11.998 g of sodium phosphate monobasic anhydrous (NaH_2PO_4, solid crystals, 99% purity, Fisher BioReagents, CAS 7558-80-7) were added to 1.0 L of distilled water and shaken until completely dissolved. Approximately 3.9 g of sodium hydroxide pellets (NaOH, 99% purity, Fisher Scientific, CAS 1310-73-2) were added in a stepwise fashion (approximately 0.5 g at a time, shaken thoroughly after each addition) to achieve pH = 8. pH was confirmed with Hydrion Insta-Chek paper. For preparation of each of the other samples, the buffer was subsequently diluted to 10^{-2} M with distilled water to 500 mL. The 0.0 mM sample of each compound simply consisted of 450 mL of 10^{-2} M sodium phosphate buffer.

Frontalin and *Z*7-12:Ac standards in liquid form were obtained from Contech Interprises, Inc. (Victoria, BC). Frontalin standards were 98% pure, and *Z*7-12:Ac standards were 96% pure. For the first experiment, the 10^{-1} mM samples of each compound were measured using a micropipette and diluted to 500 mL (this required 7.2 μL of frontalin, or 12.85 μL of *Z*7-12:Ac). From each of these, lower concentrations were prepared via stepwise dilutions, as follows: 50 mL were removed from the 10^{-1} mM sample and added to 450 mL of 10^{-2} M phosphate buffer. The sample was shaken 50

times before being used to make the next sample. 50 mL of the last sample in the serial dilution (10^{-4} mM of either compound) was removed after mixing to ensure each sample was 450 mL. To prepare the 1.0 mM vanillin samples for the first experiment 68.4 mg of vanillin crystals ($C_8H_8O_3$, 99% purity, Aldrich Chemical Company, CAS 121-33-5) were mixed with 450 mL of 10^{-2} M phosphate buffer.

In the second experiment, the highest concentrations of each compound (2.0, 1.0, and 10^{-2} mM) were measured directly for each sample using a micropipette. The 10^{-5} and 10^{-7} mM samples were prepared using stepwise dilutions from the 10^{-2} mM sample using a similar protocol as described above in the first experiment. Vanillin was not used during the second experiment to limit the number of samples present during each bioassay.

After samples were prepared in 500 mL glass bottles with Teflon-lined caps, they were wrapped in aluminum foil to conceal the identity of the samples. For bioassays, each bottle in a set was labeled with a random letter (A – F, obtained and assigned from a random number generator) so that observers were unaware of the contents of each bottle during bioassays. Samples were stored out of direct sunlight at room temperature until bioassays occurred.

Bioassay protocol

In the first experiment, bioassays consisted of three consecutive days of 1-hr observations for each compound's range of concentrations (0.0 – 10^{-1} mM). During the first experiment, elephants were observed for two 1-hr observation sessions to ascertain each elephant's 'normal' behavior and movement patterns (additionally, pre- and post-bioassay observations were conducted to evaluate the efficacy of these compounds as

olfactory enrichment, as described in Chapter 3). Each compound set was only assayed once during a 1-hr bioassay for the second experiment, and time did not permit preliminary observations before samples were poured during this phase. Additionally, two of the facilities at which samples were assayed during the second experiment were also included in the first experiment; at these facilities I was more experienced with optimal sample placement and the daily schedule that would work best. Bioassays for both experiments were conducted in exhibit yards that were familiar to each elephant, and in social groups to which each elephant was accustomed (social grouping remained consistent for each elephant across each bioassay). Each compound set was assayed separately in each experiment. On days when both frontalin and *Z*7-12:Ac sets were assayed, the compound that was presented first alternated between each elephant group tested (Table 2.1).

Each of six samples in a set were poured on the ground ≥3 m from each other to prevent cross-comparison of samples within a trunk length; substrate varied by each facility, but within a facility samples were placed on a common substrate (e.g. loose sand, packed dirt, unpolished concrete; Figure A1.1). Samples were poured by one of two observers, although the identity of each sample to both observers was unknown at the time of observation—each sample was largely odorless and colorless, making identification almost impossible when pouring samples. Location of each sample (identified by the randomly assigned letter) was marked on a map that was available during the bioassay to the observer, and when possible, samples were placed in proximity to visible landmarks (e.g. fence posts, rocks, sticks, etc.). The location of samples often corresponded with the location of desirable resources (e.g. food, water, shade) and

frequently varied between consecutive days with these resources; this increased the likelihood that focal elephants would visit the samples. The amount of food, water, and enrichment items (except for any olfactory enrichment) did not differ from routine applications. Elephant keepers, handlers, or managers were not present for bioassays, but sometimes provided direction as to the best locations to pour chemical samples based on their own experience.

Typically, two elephant groups (e.g. a solitary male, female group, pair, etc.) were assayed at each facility. The first elephant group observed was randomly assigned the first compound (frontalin or *Z*7-12:Ac) to which they were exposed, and the second group received the other compound first (Table 2.1). Morning bioassays (one of each compound; $n = 90$) occurred at 09:04 ± 65 min (mean ± SD), and afternoon bioassays occurred at 12:34 ± 105 min ($n = 68$). The average start time of the first bioassay involving *Z*7-12:Ac occurred at 09:02 ± 59 min, and the second *Z*7-12:Ac bioassay started at 12:55 ± 99 min. The average start time of the first bioassay involving frontalin occurred at 08:19 ± 52 min, while the second frontalin bioassay started at 11:33 ± 115 min.

The study design involved simultaneous (as opposed to sequential) presentation of samples poured on the ground—this allowed the elephants to naturally encounter each sample (responses were not prompted by the presence of keepers or researchers), and also gave the elephants the opportunity to compare samples to each other. Additionally, simultaneous presentation provided controls for independent variables, such as time of day, weather conditions, and food availability.

Observations took place in close proximity to elephants in order to observe fine trunk movements directed towards each sample, but they took place in areas that elephants were accustomed to seeing unfamiliar people to discourage orientation or attention towards observers. Bioassays began when all focal elephants were present in the same space as the samples and husbandry staff had left the area. All-occurrence sampling of chemosensory and accessory behaviors directed towards the samples occurred for 1 hour for all elephants in a group (Altmann, 1974; Table 2.2). Behavioral sampling began when elephants were within proximity (≤1 body length) of any sample, and behaviors were further distinguished when they occurred within 1 trunk length of the sample. Of particular interest were chemosensory behaviors described previously by Schulte and Rasmussen (1999): sniff, check, place and flehmen (Figure 2.1). One observer was responsible for recording behaviors described in Table 2.2 on paper at the time of each bioassay, while another observer recorded state behaviors described in Chapter 3 (state behaviors were only recorded during experiment 1 due to the availability of assistance during observations). Temperature at the beginning of the bioassay was also noted. Observations were recorded on video for review if necessary. After each bioassay, samples were removed by washing away samples with water or by digging up substrate.

Ethics statement

The procedures described herein were approved by the Western Kentucky University Institutional Animal Care and Use Committee (IACUC; #14-20). Additionally, a research committee at each elephant facility approved all protocols before

bioassays began. This project (including its procedures) was endorsed by the Association of Zoos and Aquariums' Elephant Taxon Advisory Group.

Statistical analyses

During bioassays, some samples were not visited by a given elephant (i.e., the elephant did not get within a trunk length of a sample). These data were excluded from all analyses. Behaviors were converted into rates (number of behaviors per hour); in some instances, bioassays lasted shorter or longer than an hour for management or husbandry purposes. Any time during the bioassays that an elephant was not present in the testing arena, or time during which an elephant was under stimulus control by a handler, was excluded from analyses. Standardizing behaviors to a rate allowed for direct comparison across bioassays.

Besides sex, I also hypothesized that concentration-dependent responses would be affected by the reproductive experience of the receiver. Elephants in captivity experience different lives than their wild counterparts, including increased nutrition and unnatural social housing conditions (Schulte, 2000). Because of this, the reproductive biology of male and female captive elephants is likely quite different than wild elephants (Keele, 2015; LaDue, Scott, & Margulis, 2014). Various sources (e.g. Moss, 1996; Shoshani, 1992) have suggested biologically relevant age classes for wild elephants that best characterize ontogenetic lifestyle shifts, and Greenwood et al. (2005) described age-related changes in chemosensory behavior of males towards frontalin. In an attempt to best describe changes in receiver behavior due to reproductive experience, males and females were characterized as 'experienced' or 'inexperienced' based on the following criteria: males that were known to have been through a complete musth cycle were

categorized as ‘experienced,’ and males that had not been through a complete musth (including one male that was castrated) were ‘inexperienced.’ For males of unknown musth history, those that were older than 30 years were ‘experienced.’ Nulliparous females, or those that had only given birth through artificial insemination, were categorized as ‘inexperienced;’ the rest of the females were categorized as ‘experienced.’

To simplify analyses, the behaviors sniff, check, place, and flehmen were combined into a single chemosensory behavior as listed in Table 2.2. Similarly, accessory behaviors were also combined into a single behavior (simply called ‘accessory behavior’).

In the first experiment, chemosensory responses to samples from each consecutive day of bioassays were compared via a mixed-model analysis of variance (ANOVA) to test if behavior differed based on repeated exposure to the samples. For each sample, only elephants that visited the sample over three consecutive bioassays were included in this analysis. The fixed effects factor was concentration, while the random effects factors were the identity of each elephant day of bioassay (first, second, or third; nested in facility). All of the concentrations ($0.0 – 10^{-1}$ mM) of both compounds were combined for this analysis, but they were tested separately from vanillin samples. Any significant differences between days were found using a Tukey’s honest significant difference (HSD) test.

The rate of chemosensory behavior towards samples shared between both experiments (the 0.0 and 10^{-2} mM samples of each compound) were compared with two-sample *t*-tests to connect the two experiments. Sexes were combined during these analyses, as overall response rates towards the samples were of interest, and not

differences between the sexes for each compound. Each sample of each compound was analyzed separately.

To test for a correlation between rates of chemosensory and accessory behavior for each frontalin and *Z*7-12:Ac concentration, a Pearson product moment correlation was used; 0.0 mM samples of both compounds were not included in these analyses because of low response rates.

An ANOVA was used to compare rates of response between concentrations based on sex and reproductive experience, and significant differences between samples were found using Tukey's HSD tests. Thresholds for any category (i.e. sex, reproductive experience) were determined by finding the response to the lowest concentration that was significantly higher than the response to the 0.0 mM sample of either compound.

The relative frequencies of chemosensory behaviors (sniff, check, place, and flehmen) were compared across concentrations of both compounds for males and females separately. Only samples to which an elephant directed chemosensory behavior were included in these analyses.

Statistical significance was set at $\alpha = 0.050$ for all analyses. All analyses were carried out using R statistical software version 3.2.3.

Results

Chemosensory responses after sequential presentations

In the first experiment, chemosensory responses did not differ significantly based on the identity of the elephant ($F_{9,42} = 0.325$, $P = 0.962$) or between the three consecutive treatment days for any of the samples (0.0 mM – 10^{-1} mM) of either compound ($F_{9,77} =$

0.556, $P = 0.829$). Therefore, for subsequent analyses, responses across the three days were averaged for each compound. Chemosensory responses to the 1.0 mM vanillin decreased progressively over the three days: although no statistical significance was observed in the presence of frontalin ($F_{2,50} = 2.781$, $P = 0.072$), elephants were significantly more responsive to vanillin in the presence of *Z*7-12:Ac on the first day than on the third day ($F_{2,29} = 4.059$, $P = 0.028$) (Figure 2.2).

Comparing the two experiments

Assaying the 0.0 mM and 10^{-2} mM samples of each compound in both experiments allowed for comparison of responses between experiments (Figure 2.3). Rates of chemosensory behavior directed towards the 0.0 mM samples of either compound did not vary significantly (frontalin: $t = 1.050$, df = 67, $P = 0.297$; *Z*7-12:Ac: $t = 0.393$, df = 58, $P = 0.696$). However, chemosensory responses by Asian elephants to the 10^{-2} mM samples were significantly higher during the second experiment for frontalin ($t = -4.419$, df = 72, $P < 0.001$) and *Z*7-12:Ac ($t = -2.863$, df = 68, $P = 0.006$). Therefore, for subsequent analyses that compare responses to various concentrations, responses to 0.0 mM samples from both experiments were combined, while responses to 10^{-2} mM samples were kept separate by experiment.

Frequencies of chemosensory and accessory behaviors

The most frequently occurring chemosensory behavior towards 10^{-7} mM – 2.0 mM samples in both frontalin and *Z*7-12:Ac bioassays was ‘sniff,’ followed by ‘check,’ ‘place,’ and ‘flehmen’ (Figure 2.4). Chemosensory behaviors occurred more frequently than accessory behaviors. For both frontalin and *Z*7-12:Ac, ‘horizontal sniff’ occurred at a rate greater than 0.30 behaviors/hr, ‘accessory trunk’ occurred at a rate greater than

0.25 behaviors/hr, ‘blow’ and ‘dust’ occurred at rates greater than 0.10 behaviors/hr, and ‘periscope sniff,’ ‘back up,’ ‘defecate,’ ‘palatal pit,’ ‘penis,’ ‘suck,’ and ‘urinate’ occurred between 0.01 and 0.10 behaviors/hr (Figure 2.5). Other accessory behaviors occurred at a rate less than 0.01 behaviors/hr. Rates of chemosensory and accessory behavior were correlated for both frontalin ($r = 0.526$, $n = 314$, $P < 0.001$) and *Z*7-12:Ac ($r = 0.598$, $n = 286$, $P < 0.001$).

Concentration effects and detection thresholds for males and females

Chemosensory responses toward frontalin samples changed significantly based on concentration for both males ($F_{9,17} = 8.475$, $P < 0.001$) and females ($F_{9,32} = 8.134$, $P < 0.001$) (Figure 2.6). Based on chemosensory responses, the detection threshold of frontalin for males was 10^{-2} mM ($P = 0.003$ for the sample presented during the second experiment; Table 2.3a), but the chemosensory response between the 0.0 mM sample and the 10^{-1} mM sample did not differ significantly ($P = 0.125$). The frontalin detection threshold for females was lower than for males: chemosensory response to the 10^{-4} mM sample was significantly higher than to the 0.0 mM sample ($P = 0.001$; Table 2.3b). Males and females showed a general increase in chemosensory response rate to frontalin samples with increasing concentration.

Both males ($F_{9,17} = 7.028$, $P < 0.001$) and females ($F_{9,31} = 10.670$, $P < 0.001$) exhibited significantly different chemosensory responses to *Z*7-12:Ac samples based on concentration (Figure 2.7). The detection threshold for males was 10^{-5} mM ($P = 0.048$; Table 2.4a), while it was 10^{-3} mM for females ($P = 0.014$; Table 2.4b). Similar to frontalin, there was a trend in increasing chemosensory response with concentration observed among males and females.

For frontalin, there was also a significant concentration effect for accessory behavior observed in both males ($F_{9,17} = 10.160$, $P < 0.001$) and females ($F_{9,29} = 2.462$, $P = 0.032$) (Figure 2.8). Male accessory responses were only significantly higher from the 0.0 mM sample at 2.0 mM, and the accessory response rate towards the 2.0 mM was also significantly higher than any other sample (Table 2.5a). Female accessory behavior towards frontalin was exhibited at a higher rate than the 0.0 mM towards the 10^{-3} mM, 10^{-2} mM (during the second experiment), 1.0 mM, and 2.0 mM samples; responses to the 10^{-7} mM and 10^{-5} mM samples also differed from the 2.0 mM sample (Tables 2.5b). For *Z*7-12:Ac, accessory behavior exhibited by males ($F_{9,143} = 5.220$, $P < 0.001$) and females ($F_{9,178} = 3.230$, $P = 0.001$) differed significantly by concentration (Figure 2.9). Male accessory behavior directed towards the 1.0 mM and 2.0 mM samples differed from the 0.0 mM sample (Table 2.6a), while female accessory responses only differed significantly between the 0.0 mM and 10^{-1} mM samples (Table 2.6b).

Effect of reproductive experience on concentration-dependent chemosensory responses

Males that were reproductively experienced ($F_{9,2} = 84.350$, $P = 0.012$) and inexperienced ($F_{9,6} = 8.654$, $P = 0.008$) showed significantly different chemosensory responses to increasing concentrations of frontalin (Figure 2.10). The detection threshold of frontalin was lower for experienced males (10^{-2} mM; Table 2.7a) than for inexperienced males (1.0 mM; Table 2.7b). A significant concentration-dependent chemosensory response pattern was also observed in both reproductively experienced ($F_{9,4} = 12.395$, $P = 0.014$) and inexperienced ($F_{9,18} = 5.896$, $P < 0.001$) females (Figure 2.11). Additionally, experienced females detected frontalin samples as low as 10^{-4} mM (Table 2.8a); inexperienced females had a higher threshold of 10^{-2} mM (Table 2.8b).

Similarly to frontalin, reproductively experienced ($F_{9,2}$ = 31.667, P = 0.031) and inexperienced ($F_{9,5}$ = 7.861, P = 0.018) males exhibited significant concentration-dependent chemosensory responses to *Z*7-12:Ac (Figure 2.12). Experienced males were capable of detecting the lowest concentration (10^{-7} mM; Table 2.9a), while inexperienced males successfully detected the 10^{-5} mM *Z*7-12:Ac samples (Table 2.9b). Reproductively experienced females did not show concentration-dependent responses to *Z*7-12:Ac ($F_{9,4}$ = 3.480, P = 0.121), while inexperienced females did ($F_{9,18}$ = 7.101, P < 0.001; Figure 2.13). Inexperienced females detected *Z*7-12:Ac samples as low as 10^{-3} mM (Table 2.10).

Changes in relative frequencies of chemosensory behavior with signal concentration

Males and females exhibited changes in chemosensory behavior with increasing frontalin concentration (Figure 2.14). Sniffs comprised 85% of male and 76% of female chemosensory responses to the 10^{-7} mM frontalin samples, while they made up only 47% and 66% of chemosensory responses to the 2.0 mM samples. There was a general increase in the relative frequencies of checks (increased 20% for males and and 7% for females) and places (increased 13% for males and 3% for females) with increasing frontalin concentration from 10^{-7} mM to 2.0 mM. Flehmens occurred as low as 10^{-3} mM for males, and 10^{-4} mM for females.

Patterns in the relative frequencies of chemosensory responses to *Z*7-12:Ac mirrored those to frontalin (Figure 2.15). Among males, sniffs comprised 86% of chemosensory behaviors to 10^{-7} mM *Z*7-12:Ac samples and 51% to 2.0 mM samples. Females exhibited a similar pattern: chemosensory responses consisted of 81% sniffs to 10^{-7} mM *Z*7-12:Ac samples and 65% to 2.0 mM samples. From 10^{-7} mM to 2.0 mM,

checks increased from 12% to 30% of total chemosensory responses in males and from 19% to 31% in females; over the same range, places increased to 15% of total chemosensory responses in males (from 2%) and 3% in females (from 0%). Both males and females exhibited flehmen responses as low as 10^{-4} mM to *Z*7-12:Ac. Females only performed flehmens to the 10^{-4} mM, 10^{-3} mM, and 10^{-2} mM *Z*7-12:Ac samples that were only present during the first experiment, while male flehmens increased from 0.5% at 10^{-4} mM to 5% at 2.0 mM.

Discussion

Asian elephants in this study showed concentration-dependent responses to both frontalin and *Z*7-12:Ac, and detection thresholds were dependent upon signal identity, and the sex and reproductive experience of the receiver. According to signaling theory, animals should behave optimally towards signals based on their perceived relevance (Alberts, 1992). Therefore, the patterns of response to chemical signals over various concentrations, and the concentration at which they are first perceived, should vary with various characteristics of the receiver such as sex and reproductive experience (Roelofs, 1978).

Frontalin is considered to be a relevant signal to both males (as a way to diffuse intrasexual competition for breeding access) and females (as a means to synchronize reproduction) (Rasmussen & Greenwood, 2005). In the present study, male and female Asian elephants showed concentration-dependent responses to frontalin in terms of both chemosensory and accessory behavior. The detection threshold of this compound based on chemosensory behavior was lower for females (10^{-4} mM) than for males (10^{-2} mM),

indicating that females are more sensitive to frontalin. However, males consistently exhibited higher rates of chemosensory behavior to almost all frontalin concentrations, and males performed chemosensory behavior to the 2.0 mM samples significantly more than the 1.0 mM samples—this was not observed in females. Reproductively inexperienced females had a higher threshold for frontalin (10^{-2} mM), and it was even higher for inexperienced males (1.0 mM). Taken together, it appears that frontalin is meaningful to both males and females over a range of concentrations, although lower concentrations are likely more relevant to females than males. Females exist in matrilineal groups separately from males, who live singly or in small bachelor groups (Eisenberg, McKay, & Jainudeen, 1971). It is likely advantageous for females to detect even small amounts of frontalin—which signals a male's intent to reproduce—because females only come into estrus every 14 to 16 weeks (Rasmussen & Greenwood, 2003). The need for males to communicate reproductive status to other males may not be as important in this regard, as illustrated by their higher threshold for frontalin. Additionally, it may be necessary for females to have previous experience around males to form a functional connection with frontalin: in the present study, females with reproductive experience had a lower detection threshold (10^{-4} mM) than females who had not been around males (10^{-2} mM). It is unclear whether this response is mediated via experiential learning, physiological mechanisms (i.e. hormones), or some other process. It would be rather unusual for an adult female in the wild to have no experience with males; nulliparous females in captivity that have been solely housed with female conspecifics would make a good comparison to pre-pubescent females in the wild to further study this phenomenon. Adult males exude higher concentrations of frontalin

during musth as they age (Greenwood et al., 2005). Because younger, less experienced males have been reported to be warier of secretions from older males, perhaps it is not surprising that reproductively inexperienced males had a higher threshold than experienced males and showed the most chemosensory behavior towards the samples of highest concentrations (Rasmussen et al., 2002). Furthermore, the simultaneous presentation of the high concentrations (1.0 mM and 2.0 mM) with the low concentrations (10^{-7} mM and 10^{-5} mM) in this study allowed for comparison between samples. The response to low concentrations of frontalin by inexperienced males may have been artificially understated as a result. Some mammals utilize multiple scent marks to communicate presence, especially in species that occupy distinct territories (Alberts, 1992; Gorman & Mills, 1984; MacDonald, 1985). However, Asian elephants are not known to defend territories (Fernando et al., 2008; Sukumar, 1991), but depositing multiple samples may help to locate potential mates, especially in areas of high population density.

*Z*7-12:Ac has been described as a primarily female-to-male signal (Rasmussen & Greenwood, 2005; Rasmussen et al., 2005; Schulte, Freeman, Goodwin, Hollister-Smith, & Rasmussen, 2007), and the present study supports this role based on the sex-dependent detection thresholds. Here, compared to the males, female elephants exhibited less chemosensory behavior to *Z*7-12:Ac. Females successfully distinguished *Z*7-12:Ac at a mid-range concentration (10^{-3} mM), while reproductively experienced males were able to detect samples at the lowest concentration presented (10^{-7} mM) and inexperienced males at the second lowest concentration (10^{-5} mM). In this study the *Z*7-12:Ac detection threshold for experienced males was not established. Additionally, there was less of a

concentration-dependent chemosensory response observed among females (and it was statistically absent among reproductively experienced females). Presumably, some of the reproductively experienced females in this study were cycling and producing *Z*7-12:Ac themselves (Brown, Olson, Keele, & Freeman, 2004), which may have contributed to the lack of concentration-dependent responses. The synthetic compounds presented in the bioassays may not have been of interest compared to their own endogenous signals, especially since they were applied in sodium phosphate buffer and not in urine. Younger, less experienced females may have been more interested in *Z*7-12:Ac as a signal of intrasexual competition; younger females are subordinate to older females, which has been shown to affect behavior in captive Asian and African elephants (Freeman, Weiss, & Brown, 2004; Freeman, Schulte, & Brown, 2010).

Thresholds of various sensory modalities have been shown to vary with ontogenetic lifestyle shifts in a variety of vertebrate species (e.g. Apfelbach, Russ, & Slotnick, 1991; Borg, 1982; Carvalho, Noltie, & Tillitt, 2002; Ruben, 1992; Wright, Higgs, & Leis, 2011). Under signaling theory, it is logical that reproductive experience (as a sort of proxy for age) should influence the thresholds at which sex pheromones such as frontalin and *Z*7-12:Ac are first detected. Here, I showed that the threshold for each compound varies with both sex and reproductive experience, in the theoretical context of either signal's assumed relevance.

I was not able to determine a concentration of maximum chemosensory response of either frontalin or *Z*7-12:Ac for males, as there was a generally increasing trend with concentration, and the mean response to the 2.0 mM sample was significantly higher than to the 1.0 mM sample of each compound. However, reproductively experienced males

did not differ in their chemosensory response to the 1.0 mM and 2.0 mM samples of either frontalin or *Z*7-12:Ac, indicating that they may be responding maximally at 1.0 mM. Similarly, females of any reproductive experience exhibited chemosensory behavior at similar rates for the 1.0 mM and 2.0 mM samples of both frontalin and *Z*7-12:Ac, although future studies should investigate higher concentrations to elucidate response trends.

Signaling theory predicts that it is inherently costly to respond more intensely to signals beyond a certain concentration; indeed, in any circumstance it is theoretically impossible to exhibit a greater or more frequent response above a maximum level (Grafen, 1990; Killeen, 1975; Roelofs, 1978). The finding that reproductively inexperienced males appear to respond to higher concentrations of either compound more frequently than females or experienced males may indicate that it is advantageous to respond to these signals at any concentration with which they can be detected. This age-related pattern is in accordance with findings by Bagley (2004), who showed that younger male African elephants were more investigative than older males. Scott (2002) also found that subordinate and nonmusth male Asian elephants could successfully differentiate between musth and nonmusth urine, pointing to the notion that reproductive signals are of interest to even inexperienced males. While this study did not find differences in maximum chemosensory response rates between females of different reproductive experience, younger African elephant females have been reported to exhibit higher rates of chemosensory behavior than older females (Loizi, Goodwin, Rasmussen, Whitehouse, & Schulte, 2009). It was cost-prohibitive to run bioassays with higher concentrations than 2.0 mM, so it is unclear if maximum response rates were achieved in

any demographic group with either compound, and if not, what concentrations yield maximum responses.

The process of olfactory habituation may be related to the perceived biological relevance of the odor (Daniel & Derby, 1988; Mandairon, Stack, Kiselycznyk, & Linster, 2006). In the first experiment with three consecutive days of bioassays, elephants did not exhibit decreasing responses to either frontalin or *Z*7-12:Ac after three days of repeated exposure, like they did to vanillin. Captive African elephants (*L. africana*) exposed to novel auditory stimuli have been shown to habituate rather quickly to noise, decreasing their distress, avoidance, and vigilance responses (Goodyear & Schulte, 2015). A similar habituation to vanillin was likely occurring here (Raderschall, Magreath, & Hemmi, 2011), as vanillin is thought to be biologically irrelevant to Asian elephants. Habituation to chemical cues has been described in mountain beavers (*Aplodontia rufa*, Epple et al., 1995), deer (*Cervus elaphus* and *Capreolus capreolus*, Elmeros, Winbladh, Andersen, Madsen, & Christensen, 2011), and bank voles (*Clethrionomys glareolus*, Ylönen, Eccard, Jokinen, & Sundell, 2006), and even in arthropod taxa such as freshwater isopods (*Lirceus fontinalis*, Holomuzki & Hatchett, 1994) and mosquito larvae (*Culiseta longiareolata*, Roberts, 2014). Furthermore, these findings support the role of both of these compounds as pheromones because pheromones have been classically defined by their ability to yield repeated responses.

Elephants showed higher chemosensory responses towards the 10^{-2} mM samples of both frontalin and *Z*7-12:Ac in the presence of high and low concentrations (the second experiment) compared to mid-range concentrations (the first experiment), but responses to the 0.0 mM samples were the same across both experiments. While this

study focused on the releaser effects of these compounds (that is, the immediate behavioral responses of elephants to frontalin and *Z*7-12:Ac; Albone, 1984), it is also possible that these compounds have primer effects (Wilson & Bossert, 1963). Primer effects are those that involve longer lasting physiological changes, which may induce concomitant behavioral changes (e.g. Ekerholm & Hallberg, 2005; Ferkin, 1999). Neither frontalin nor *Z*7-12:Ac has been investigated for any primer effects in Asian elephants, although it is conceivable that the detection of exceedingly high (1.0 or 2.0 mM) or low (10^{-5} or 10^{-7} mM) concentrations physiologically stimulated elephants to investigate other samples (e.g. 10^{-2} mM) more intensely. Alternatively, the presence of these samples may have simply changed the context of the environment in a way that made elephants more sensitive to the 10^{-2} mM samples (Hager & Teale, 1994; Vet, 1999). For instance, high concentrations of either compound may have signaled that a reproductively active individual was in the area—stimulating the receiver to actively search for more samples—or investigate previously visited samples more closely, to confirm the message.

Rates of chemosensory behavior were correlated with accessory behavior for both frontalin and *Z*7-12:Ac. Similarly, Bagley et al. (2006) found that male African elephants exhibited high rates of accessory trunk behavior (i.e. blow, flick, pinch, suck, and wriggle) with chemosensory behavior towards follicular urine from females after the second LH surge. These behaviors, which largely consisted of horizontal sniffs and trunk movements, may serve to clear the trunk or isolate the headspace of volatile signals (Schulte et al., 2005). In the present study, rates of accessory behavior (trunk and body, Table 2.2) were lower than chemosensory behavior across all concentrations of both

compounds for males and females. *Z*7-12:Ac is not a highly volatile compound itself, and it is bound to albumin in urine, further reducing its volatility (Lazar, Rasmussen, Greenwood, In-Soek, & Prestwich, 2004; Rasmussen, Lazar, & Greenwood, 2003). Although not as well known, it is expected that the volatility of frontalin is higher than *Z*7-12:Ac because it is exuded from the temporal glands and wafted via ear-flapping to potential receivers (Perrin, Rasmussen, Gunawardena, & Rasmussen, 1996; Poole, 1997).

Elephants detect chemical signals through the main olfactory and vomeronasal systems, which involve both tactile and olfactory reception (Schulte & Rasmussen, 1998). Tactile interactions with samples (i.e. chemosensory behaviors like 'checks' and 'places') may indicate increased interests in signals. While sniffs were the dominant chemosensory behaviors exhibited towards both frontalin and *Z*7-12:Ac across all concentrations for both sexes, there was a general trend in increasing frequencies of checks and places with increasing concentration, perhaps supporting the idea that there is greater interest in samples of higher concentration. A similar pattern was observed for male flehmens with both compounds, and while females exhibited flehmens towards most of the high concentrations of frontalin (starting as low as 10^{-4} mM), females only performed flehmens towards lower concentrations of *Z*7-12:Ac (10^{-4} mM, 10^{-3} mM, and 10^{-2} mM). Because flehmen responses have been suggested to correspond to relative reproductive interest of a receiver to chemical signals (Perkins & Fitzgerald, 1992; Schulte et al., 2005), the pattern of flehmens observed here contributes to our understanding of the relevance of these signals across sex and reproductive experience as they vary with concentration.

Low concentrations of *Z*7-12:Ac were hypothesized to be largely irrelevant to females, and so it is surprising that they exhibited flehmens to only low—but not high—*Z*7-12:Ac concentrations; flehmens are considered to be a strong chemosensory response in *E. maximus* (Schulte & Rasmussen, 1999). Female Asian elephants in the follicular phase have been reported to preferentially respond to follicular- over luteal-phase urine, presumably as a mechanism to monitor estrous dynamics within an asynchronous female group (Slade, Schulte, & Rasmussen, 2003). Because *Z*7-12:Ac is at its highest concentration in female urine near the end of the follicular phase, the results presented here appear to disagree with the study by Slade et al. (2003). However, the estrous statuses of only some of the females in this study were known, and even so, flehmens comprised less than 3% of chemosensory responses to these *Z*7-12:Ac samples and reproductively experienced females showed no differences in chemosensory behavior based on signal concentration. Further investigation into intrasexual chemical signaling in Asian elephants (especially in wild populations with limited resources) that better analyzes the effect of female reproductive status on the detection of *Z*7-12:Ac at low and high concentrations is warranted.

A logical extension of this project is to more closely investigate detection thresholds using conditioned discrimination trials similar to those conducted with biologically irrelevant chemicals by Arvidsson et al. (2012) and Rizvanovic et al. (2013). It is likely that Asian elephants can detect concentrations lower than what is reported here if they are trained to do so. Simultaneous bioassays allow for various environmental variables (e.g. weather, time of day, substrate) to be standardized within individual animals, but animals may also respond in a context-dependent fashion based on the

presence of other chemical samples. Schellinck, Rooney, and Brown (1995) showed that rats responded only to certain odors in discrimination trials, but not in bioassays similar to those presented here. However, the ecological relevance of responses in discrimination trials (or even sequential bioassays) is questionable (Wolff, 2003). In this case, lower concentrations of frontalin or *Z*7-12:Ac may extend below the range of what is biologically meaningful, and responses may simply be artifacts of experimental design. Still, there is interest in the chemosensory abilities of elephants, especially given the rather large olfactory receptor gene repertoire of the African elephant (Niimura et al., 2014). The ability of reproductively experienced males to respond to *Z*7-12:Ac samples as low as 10^{-7} mM is certainly remarkable.

CHAPTER 3

EVALUATING THE EFFICACY OF CHEMICAL SIGNALS AS ENRICHMENT FOR CAPTIVE ELEPHANTS

Introduction

Elephants maintained in captive facilities (in zoos, circuses, wild animal parks, etc.) experience very different lives from their wild counterparts. Given their exceptional cognitive abilities, long lifespans, and large size, it can be difficult to ensure that the physical and psychological demands of elephants are met in captivity. It is probably unwise to use nature as a gauge with which to measure the welfare of captive elephants, given the habitat variability and chronic stress that many wild elephants experience (Hutchins, 2006). Welfare assessments have traditionally focused on a resource-based approach, whereby the physical needs of an animal were compared to the status of the rest of the captive population (Mellen & MacPhee, 2001; Whay, 2007). However, zoo animal managers are progressively moving towards animal-based assessments that are centered on objective measures of animal welfare (e.g. stress hormones, health indicators, keeper surveys, etc.). The principal benefit of an animal-based approach is that the instantaneous welfare status of an individual animal can be compared against itself, yielding a more tailored approach to captive animal management. Still, it can be difficult to recognize an individual animal's physical and behavioral needs. A goal-oriented approach to environmental enrichment—that is, one that involves setting goals for a particular enrichment program and evaluating the enrichment's efficacy in light of those goals—may be particularly effective for management strategies that focus on animal-based assessments.

Environmental enrichment serves to improve the physical and psychological welfare of captive animals (Swaisgood & Shepherdson, 2005). Many published elephant enrichment studies have focused on varying diet, feeding schedule, or food presentation (Elzanowski & Sergiel, 2006; Gloyns & Plowman, 2000; Morimura & Ueno, 1999; Rees, 2009; Sjöberg, 2011; Stoinski, Daniel, & Maple, 2000; Wiedenmayer, 1998). Food-based enrichment is relatively easy to implement, but it is unclear whether there are any causative, long-lasting behavioral effects. Even though elephants spend a large portion of their time foraging, it may be more effective to implement enrichment that better engages their sensory systems (Wells, 2009). Captive environments are often void of many sensory stimuli, despite the elaborate sensory systems that elephants possess.

Sensory enrichment can be relatively cost-effective and yield behavioral effects that are longer lasting. Auditory stimulation has been shown to exhibit lower rates of undesired stereotypic behavior in Asian elephants (Wells & Irwin, 2008). Many mammal species are scent-oriented, and so chemical signals may be good targets for sensory enrichment programs (Swaisgood & Schulte, 2010). Despite this, few studies have systematically evaluated the efficacy of scent-based enrichment (Hoy, Murray, & Tribe, 2010). There are inherent obstacles to implementing olfactory enrichment—namely identifying relevant odors and presenting the odors in an appropriate fashion—that make evaluation of scent-based enrichment challenging (Clark & King, 2008). Still, there is growing appreciation for the potential of chemical signals to increase the behavioral diversity of captive animals. For example, captive African wild dogs (*Lycaon pictus*) responded to chemical cues from natural prey species—but not odors from competitors or unnatural prey—by performing higher frequencies of affiliative, submissive, and

dominant behaviors (Rafacz & Santymire, 2014). Captive black-footed cats, *Felis nigripes*, increased their time spent engaging in active behavior when exposed to novel odors (Wells & Egli, 2004). Furthermore, Martínez-Macipe, Lafont-Lecuelle, Manteca, Pageat, and Cozzi (2015) showed that captive lions (*Panthera leo*) exposed to chemical signals exhibit longer periods of play and exercise. It is unclear whether these behavioral effects are a consequence of signal relevance (in this case with *P. leo*, cat facial and appeasing pheromones). Gorillas (*Gorilla gorilla gorilla*) exposed to artificial odors showed no changes in general behavior (Wells, Hepper, Coleman, & Challis, 2007); primates are generally not scent-oriented, although the irrelevance of these artificial odors likely contributed to these results. Further investigation into the relationship between the apparent biological relevance of a chemical signal and its effectiveness as enrichment is certainly warranted.

In the wild, male and female elephants live almost exclusively separate lives: females form larger matrilineal herds comprised of related females and their offspring, and upon reaching puberty males leave their natal groups to form smaller bachelor groups or live solitarily (Archie et al., 2007; Buss, 1961; Eisenberg et al., 1971). Generally, male elephants only enter a female herd during musth when they may compete with other males for access to receptive females for breeding purposes (Eisenberg, 1980; Hollister-Smith et al., 2007; Slotow, van Dyk, Poole, Page, & Klocke, 2000; Sukumar, 2003). Historically, many captive elephant facilities have maintained their animals in substantially smaller groups, typically comprised of a few unrelated females (Schulte, 2000). It was simply easier to manage females, as captive male elephants have more robust housing requirements, and so many more female elephants comprised the North

American founder population (Keele, 2015; Olson, 2011). This sex bias is still evident today. Currently many facilities housing elephants have at least one adult male, although he is often housed separately from the females (much like what is assumed occur in natural settings). However, most captive male elephants experience no competition for breeding access, and females exhibit little (if any) mate choice. As captive breeding success increases into the future with an approximate 1:1 sex ratio at birth, there will be an inevitable shift in the demographics of the captive population, and elephant facilities will have to accommodate larger social groups with more male elephants. There is strong evidence to suggest that frontalin and *Z*7-12:Ac serve purposes of social organization in Asian elephants, and it is very likely that these signals may serve to better mediate social interactions and enhance breeding efforts (Rasmussen, 1998; Rasmussen & Krishnamurthy, 2000; Schulte et al., 2007).

Elephant chemical signals can be detected by conspecifics either through taste or through the trigeminal, main olfactory, and vomeronasal systems (Rasmussen, 2006; Rasmussen & Schulte, 1998). Evidence of the importance of chemical signals to elephants is also present in their anatomy: elephants have well-developed olfactory and vomeronasal organs, and have evolved specialized scent glands—namely the interdigital and temporal glands (Johnson & Rasmussen, 2002; Lamps et al., 2001; Rajaram & Krishnamurthy, 2003). The anatomy of elephants makes them well-suited to detect odors, and investigatory behavior (or 'interest') is affected by the physiological status of the receiver (Bagley et al., 2006; Meyer et al., 2008; Slade et al., 2003). Through the scope of a goal-oriented enrichment program, it is necessary to consider the motivations of an individual animal before implementing a chemical signal as olfactory enrichment.

Because the concentration of a signal may also affect its message (see Chapter 2), animal managers should also ensure that signals are applied appropriately.

Even though applying scents as enrichment for captive elephants is rather commonplace in zoos, the motivation for elephants to investigate these 'irrelevant' odors is questionable (Hare & Gilbert, 1994). Scents selected as olfactory enrichment are usually those that are assumed to be of interest to the animals (e.g. artificial perfumes, spices, etc.), but human perceptions are not necessarily aligned with the sensory biases of other species. Furthermore, the efficacy of various scents as enrichment are rarely evaluated systematically (Leach, Young, & Waran, 1998). To date no known studies have thoroughly investigated the efficacy of biologically relevant chemical signals as enrichment for captive Asian or African elephants of both sexes.

The purpose of this chapter was to investigate the potential for using frontalin and/or *Z*7-12:Ac as olfactory enrichment for captive Asian elephants by comparing behavior before and after their application. Similar to reports by Martínez-Macipe et al. (2015), Rafacz and Santymire (2014), and Wells and Egli (2004), I expected that these chemical signals would result in increased time spent engaging in active behaviors (e.g. walking, manipulating objects, investigating the environment, socializing) and decreased time in inactive behaviors. Frontalin and *Z*7-12:Ac serve as reproductive signals, so I hypothesized that sex and reproductive experience would also influence overall activity budgets. I also expected that these chemical signals would function in reducing stereotypic behavior after they had been applied (Hoy et al., 2010; Rushen & Mason, 2006; Swaisgood & Shepherdson, 2006).

Methods

Sample parameters

During the first experiment described in Chapter 2, behavioral data related to activity budget (i.e. state behaviors) were collected during bioassays of the mid-range concentrations ($0.0 – 10^{-1}$ mM). Specifically, the purpose of these observations was to test for broader behavioral effects that illustrate any enriching effects of these compounds for captive management purposes. This procedure was impossible to conduct during the second experiment described in Chapter 2 involving low and high concentrations because bioassays were conducted for only one day without pre- or post-evaluations. Additionally, only one observer was present for the second experiment, making simultaneous data collection impractical. The sample for this enrichment study included 50 elephants from 10 facilities across North America (two elephants that were included during bioassays were excluded from these observations due to availability of observers). Details on each elephant are provided in Table A1.1.

Chemical sample preparation

Chemical samples were prepared as described in Chapter 2. Briefly, six 450 mL samples were prepared in 10^{-2} M sodium phosphate buffer for each bioassay set: a blank buffer sample, a 1.0 mM vanillin sample, and a set of four concentrations (10^{-4} mM, 10^{-3} mM, 10^{-2} mM, and 10^{-1} mM) prepared via stepwise dilutions of either frontalin or *Z*7-12:Ac. Samples were prepared less than 24 hours before bioassays began in glass bottles at room temperature. Each bottle in a set was concealed and randomly assigned a letter (A – F) to conceal its identity to observers.

Observation protocol

Activity budget observations occurred simultaneously with all-occurrence sampling of chemosensory behavior. Additionally, two 1-hr observation sessions were conducted the day before bioassays began and the day after the third consecutive day of bioassays to allow for an ABA experimental design used in many studies on the behavioral effects of enrichment (Saudargas & Drummer, 1996; Swaisgood & Shepherdson, 2005; Table 2.1).

Chemical samples were put out as described in Chapter 2: samples were poured on the ground ≥3 m from each other, and the location of each sample was marked on a map available to observers. Instantaneous scan sampling of behavior with 1-min scans occurred for 1 hour (Altmann, 1974). Common state behaviors were lumped into broad categories of interest: interaction behaviors, self-maintenance behaviors, foraging, walking, inactive behaviors, and other behaviors (Table 3.1). Additionally, all-occurrence sampling of chemosensory behaviors (sniffs, checks, places, and flehmens) directed towards chemical samples were recorded on days 2, 3, and 4 as described in Chapter 2 (Table 2.2).

Males and females were designated as being reproductively experienced or inexperienced. Males that had gone through a complete musth cycle (or males of unknown musth status who were older than 30 years old) were experienced, while all other males were inexperienced. Nulliparous females or females that had given birth only through artificial insemination were inexperienced; all other females were experienced. Fifty elephants from 10 facilities were included in these analyses, consisting of 18 males (6 reproductively experienced and 12 inexperienced) and 32

females (11 experienced and 21 inexperienced). According to the latest records from the Association of Zoos and Aquariums' Asian Elephant Species Survival Plan® (Keele, 2015), there are 254 Asian elephants (55 males and 199 females) held at 55 institutions (e.g. zoos, wild animal parks, circuses, private owners) in North America. Therefore, this phase of the project included 18% of North American facilities, with 20% of the population (33% of males and 16% of females). These elephants were separated into 27 unique social groups comprising 270 hours of observations (two 1-hr observations per group during pre-treatment, three 1-hr observations for each compound over the three days of bioassays, and two 1-hr observations during post-treatment). These 270 hrs consisted of 16,200 scans (30,000 scans if each elephant is counted as an individual scan), of which 27 individual scans (0.09%) were excluded from analysis because elephants were under stimulus control by a handler.

Ethics statement

The procedures described herein were approved by the Western Kentucky University Institutional Animal Care and Use Committee (IACUC; #14-20). Additionally, a research committee at each elephant facility approved all protocols before observations began. This project (including its procedures) was endorsed by the Association of Zoos and Aquariums' Elephant Taxon Advisory Group.

Statistical analyses

Repeated measures analyses of variances (ANOVAs) were used to analyze the effects of either frontalin or *Z*7-12:Ac on the proportion of time spent engaged in each behavior on day 1 before either compound was applied (pre-treatment), during bioassays when compounds were present (treatment), and day 5 after they were removed (post-

treatment). Behaviors for the three days during which compounds were present were averaged together. Pairwise *t*-tests were used to identify significant differences between treatments.

There is evidence to suggest that behavior and physiological measures (e.g. hormones) follow circadian cycles in captive Asian and African elephants (Casares et al., 2016; Menargues, Urios, Limiñana, & Mauri, 2012; Posta, Huber, & Moore, 2013; Rees, 2009). Any behavioral differences within individuals that were caused by the compounds during the treatment phase may have been time-specific. Therefore, I only compared behavior from each treatment phase to behavior in the corresponding pre- and post-treatment phases that occurred at approximately the same time of day. That is, across five days, only behavior from observations of the same time (morning or afternoon) were compared, whether the treatment was frontalin or *Z*7-12:Ac.

Additionally, the relationship between chemosensory behavior and three behavior categories (inactivity, interaction, and walking) was analyzed. Chemosensory behavior per hour (comprised of sniffs, checks, places, and flehmens) directed towards any of the 10^{-4} – 10^{-1} mM samples of each compound was averaged across three days for each elephant; chemosensory behavior directed toward 0.0 mM samples of either compound or the 1.0 mM vanillin sample was excluded. Inactive, interactive, and walking behaviors were averaged across days 2, 3, and 4 (the days during which chemical samples were present) for each elephant. A Pearson product moment correlation was used to test for significance in the relationship between chemosensory behavior and each of the behavior categories (inactivity, interaction, and walking).

A separate analysis was conducted for stereotypic behavior; because I was interested in the ability of either compound to decrease stereotypy in the light of a goal-oriented enrichment strategy, elephants that did not exhibit stereotypy on the first day of observations were excluded from this analysis. Repeated measures ANOVAs were used again to compare the proportion of time engaged in stereotypic behavior on the day before (pre-treatment) and after (post-treatment) that frontalin and *Z*7-12:Ac were applied (one in the morning and the other in the afternoon, determined by a randomization process, see Chapter 2).

Statistical significance was set at $\alpha = 0.050$ for all analyses. For pairwise analyses of significant differences between treatments, *P*-values were corrected for multiple comparisons via Bonferroni adjustments. All analyses were carried out using R statistical software version 3.2.3.

Results

Effects of chemical samples on behavior

In general, elephants spent little time exhibiting chemosensory behavior towards chemical samples. During frontalin bioassays, chemosensory behavior only comprised 0.9 ± 0.03% (mean ± SE) of scans for males, and 0.7 ± 0.03% for females. Similarly, males exhibited chemosensory behavior during 0.9 ± 0.04% of *Z*7-12:Ac scans, while chemosensory behavior comprised 0.5 ± 0.02% of scans for females.

The time spent engaging in most behaviors over the three treatments did not significantly change with sex or reproductive experience after exposure to frontalin (Table 3.2) or *Z*7-12:Ac (Table 3.3). Reproductively inexperienced (but not experienced)

males spent significantly less time standing ($F_{2,22}$ = 0.017, P = 0.017), and more time walking ($F_{2,22}$ = 3.984, P = 0.033), during post-treatment compared to pre-treatment after frontalin was applied. In general, males exhibited stereotypy more frequently during frontalin bioassays than during the post-treatment phase ($F_{2,34}$ = 3.313, P = 0.049). Interestingly, experienced females urinated during more scans on bioassay observations than observations during either pre- or post-treatment ($F_{2,20}$ = 3.811, P = 0.028). Reproductively inexperienced females spent significantly less time drinking during post-treatment after frontalin application ($P_{2,40}$ = 4.389, P = 0.019). Regardless of reproductive experience, females spent less time out of view of visitors on the last day of observations after the frontalin samples were removed compared to pre-treatment ($F_{2,62}$ = 3.811, P = 0.028).

*Z*7-12:Ac samples did not appear to significantly alter the activity budgets of males, except that reproductively inexperienced males exhibited less social behavior during post-treatment compared to days when *Z*7-12:Ac samples were present ($F_{2,22}$ = 4.445, P = 0.024). Females foraged more during the post-treatment days compared to pre-treatment ($F_{2,62}$ = 3.236, P = 0.046). Reproductively inexperienced females investigated their environment less after removal of *Z*7-12:Ac during post-treatment ($F_{2,40}$ = 4.771, P = 0.014). 'Investigation' consisted of sniffing the air, an object, another elephant, urine, or feces, but not chemical samples (Table 3.1). Inexperienced females also groomed themselves less when *Z*7-12:Ac was present, a change that persisted into the post-treatment phase ($F_{2,40}$ = 4.223, P = 0.022). Additionally, reproductively inexperienced females walked more during *Z*7-12:Ac bioassays compared to post-

treatment ($F_{2,40}$ = 6.910, P = 0.003). Neither reproductively experienced males or females showed any significant changes in behavior when analyzed separately.

The results of the correlation tests between chemosensory behavior and behavioral states (inactivity, interaction, and walking) are summarized in Table 3.4. There was a direct correlation between rate of chemosensory behavior directed towards frontalin samples and proportion of time spent walking among reproductively experienced—but not inexperienced—females (r = 0.726, n = 11, P = 0.011; Table 3.4a). Rates of chemosensory behavior directed towards frontalin did not correlate with inactivity or interactive behaviors in reproductively experienced or inexperienced elephants of either sex. However, reproductively experienced (but not inexperienced) males that exhibited higher rates of chemosensory behavior towards the *Z*7-12:Ac samples tended to walk more (r = 0.824, n = 6, P = 0.044; Table 3.4b). There was an indirect relationship between chemosensory behavior directed towards *Z*7-12:Ac samples and inactivity exhibited by reproductively inexperienced females (r = –0.460, n = 21, P = 0.036), and a direct relationship between interactive behaviors and *Z*7-12:Ac chemosensory behavior in inexperienced females (r = 0.477, n = 21, P = 0.029). The chemosensory behavior to *Z*7-12:Ac by the Asian elephants in this study showed no correlation to either inactivity, interactive behavior, or walking observed among reproductively inexperienced males or reproductively experienced females.

Effects of chemical samples on stereotypy

Following a goal-oriented approach to enrichment, only elephants that exhibited stereotypy on the first day of observations (pre-treatment) were included in these analyses. For the frontalin observations, there were 3 males (2 reproductively

experienced and 1 inexperienced) and 5 females (2 experienced) that exhibited stereotypy during the pre-treatment observations; for the *Z*7-12:Ac observations, there were 5 males (4 experienced) and 12 females (6 experienced). Neither frontalin (male: $F_{2,4} = 4.966$, $P = 0.082$; female: $F_{2,8} = 0.408$, $P = 0.678$) nor *Z*7-12:Ac (male: $F_{2,8} = 0.614$, $P = 0.565$; female: $F_{2,22} = 0.899$, $P = 0.421$) significantly reduced stereotypy for either males or females (Figure 3.1). A similar pattern was observed for both compounds based on reproductive experience of males and females (Figure 3.2). Frontalin did not significantly reduce stereotypic behavior in reproductively experienced males ($F_{2,2} = 4.458$, $P = 0.183$) or females that were experienced ($F_{2,2} = 1.187$, $P = 0.457$) or inexperienced ($F_{2,4} = 0.216$, $P = 0.815$). Applying *Z*7-12:Ac did not result in a significant decrease in stereotypy exhibited by reproductively experienced males ($F_{2,6} = 1.304$, $P = 0.339$), experienced females ($F_{2,10} = 0.089$, $P = 0.916$), or inexperienced females ($F_{2,10} = 1.233$, $P = 0.322$). Statistical analyses on stereotypy were not conducted for reproductively inexperienced males, because there was only one inexperienced male that exhibited stereotypic behavior during pre-treatment observations of either frontalin or *Z*7-12:Ac (and it was the same male for each compound). In this case, time spent performing stereotypic behavior actually increased when samples were present. During frontalin trials, stereotypy comprised 25% of scans during the pre-treatment phase, 37% of scans when samples were present, and 15% of scans during post-treatment. A similar pattern was observed for this male during *Z*7-12:Ac bioassays: stereotypy increased from 13% of scans during the pre-treatment observation to 35% of scans when *Z*7-12:Ac was present, and decreased to 25% during the post-treatment phase.

Discussion

The elephants in this study comprised a substantial portion of the captive Asian elephant population in North America (Keele, 2015), and a variety of housing conditions (zoos, wildlife parks, private owners) were sampled. Therefore, the results of this study can likely be considered representative of the larger population. Despite the known importance of chemical signals in the social organization of Asian elephants (Rasmussen & Krishnamurthy, 2000), males and females were observed spending little time engaging in chemosensory behavior, and there appeared to be only minor effects of both compounds on the activity budgets of elephants of either sex or reproductive experience category. Still, activation of olfactory systems takes little time, and because the relevance of odors to mammalian behavior cannot be overstated, there still may be broader implications for behavior (Clark, Melfi, & Mitchell, 2005; Hancox, 1990).

A variety of behavioral changes occurred as a result of exposing Asian elephants to synthetic versions of natural chemical signals (Figure 3.3). Reproductively inexperienced males spent less time standing during post-treatment of frontalin. Males walked significantly more during frontalin bioassays (regardless of reproductive experience), but this was not statistically significant when experienced males were analyzed separately. Additionally, inexperienced males were inactive during fewer scans the day after frontalin bioassays. This suggests that optimizing the application of frontalin may have positive, enriching effects on male elephant behavior, especially in younger males. However, neither frontalin nor *Z*7-12:Ac reduced stereotypic behavior in elephants that exhibited stereotypy during the pre-treatment phase (and they may indeed cause an increase in stereotypic behavior in inexperienced males).

It is quite interesting that reproductively experienced—but not inexperienced—females urinated more when frontalin samples were present compared to pre- or post-treatment. If experienced females have formed an association between the presence of adult males and frontalin as is hypothesized, then it is possible that these females were signaling their reproductive status to potential mates (Rasmussen et al., 2005). While inexperienced females drank less during frontalin post-treatment, and females foraged increasingly more with successive *Z*7-12:Ac treatments, the biological significance of these findings is unclear. Neither of these compounds is suspected to function in resource acquisition, but applying these odors may have contributed to behavioral trade-offs that subsequently reduced time spent drinking or increased time spent foraging. Females also spent fewer scans out of view of the public. While this alone is not necessarily enriching for the elephants, it may be of interest to zoo managers.

As a compound involved in reproductive synchronization, *Z*7-12:Ac was predicted to elicit different responses from elephants based on the sex and reproductive experience of the receiver. There were almost no effects of *Z*7-12:Ac on male behavior, although inexperienced males socialized less. There were only six inexperienced males with access to conspecifics though, so this result may be an artifact of small sample size. Similar to the responses females exhibited to frontalin, females spent more time foraging—and less time engaging in self-maintenance—during post-treatment of *Z*7-12:Ac. Reproductively inexperienced females also spent less time exhibiting investigatory behavior; this finding contradicts the goal-oriented approach of olfactory enrichment, although repeated exposure to multiple samples may have diluted any single sample's efficacy as enrichment. Here, the elephants were expected to encounter

chemical samples naturally. However, because captive elephants may be exposed to chemical signals more regularly (or at least in smaller spaces where they are closer together), it may be appropriate to motivate these animals to investigate odors by pairing scent stimuli with reinforcement (e.g. food, or a task that is rewarding in itself; Inglis, Forkman, & Lazarus, 1997). Elephants may subsequently search out chemical signals without encouragement after previous experience associating a chemical signal with a reward (Inglis, Langton, Forkman, & Lazarus, 2001).

This study was designed to complement an investigation into the effects of chemical signal concentration on chemosensory response. That is, the preparation and placement of samples were not optimized for a thorough exploration of using pheromones for enrichment in Asian elephants. For instance, samples were placed close together and in proximity to the observers so that detailed observation of chemosensory behavior could occur. Additionally, repeated exposure to these compounds (three bioassays of each for three consecutive days) may have inadvertently decreased their effectiveness. Clark and King (2008) emphasized the need to carefully consider how odors are applied in terms of location, concentration, and frequency. Presumably, the elephants in this study were familiar with at least one of these compounds through access to conspecifics, either at the time of observation or through previous experience. Pairing these compounds with unfamiliar urine may have enhanced any enriching properties they might have in a novel context (Baker, Campbell, & Gilbert, 1997; Calderisi, 1997).

The paucity of findings may be interpreted hastily as a lack of enrichment potential in using biologically relevant odors like pheromones. Chemical signals similar to those tested in this study can contribute to the complexity of captive environments and

provide opportunities for the expansion of behavioral repertoires (Newberry, 1995). The human-centered aesthetics of clean environments in captivity often directly conflict with the use of odors in mammalian communication, and this effect is compounded because of human bias to target other sensory modalities for enrichment. It can also be more difficult to measure the effectiveness of odors compared to other enrichment strategies: for instance, it is relatively easy to gauge whether food-based enrichment or manipulanda has been successful by quantifying consumptive use. As we better understand how best to evaluate sensory enrichment through the scope of animal-based assessment, and simply how animals interact with and are motivated by chemical signals in general, perhaps the challenges of implementing olfactory enrichment (namely, presentation in space and time) will be overcome to make biologically relevant odors commonplace in zoos (Whay, 2007).

Both frontalin and *Z*7-12:Ac are suggested to have behavioral effects in Asian elephants (Rasmussen & Greenwood, 2003; Rasmussen et al., 2005; Rasmussen et al., 2016). After the bioassays described here, keepers were asked to report any changes they observed as a result of exposing their elephants to either of these compounds. Although only anecdotal, there is evidence that these compounds have profound effects beyond those reported already, including increasing investigatory behavior, enhancing behavioral diversity, and prolonging musth and musth-like symptoms (Table 3.5). One reproductively experienced male participated in a routine semen collection during bioassays; keepers reported his sperm quality to be exceptionally high. As previously described, this study was not designed in a way that optimally analyzed long-term changes like these. However, because the health and reproduction of much of the captive

population is closely monitored, a project that examines the primer effects of these compounds (that is, changes related to physiology and development) is easy to conceive. Ethologists have begun to recognize the value of odors in variety of applied contexts—including in agriculture, zoos, laboratories, and companion animal settings—as a way to enhance animal welfare by bolstering health measures such as reproduction (Nielsen et al., 2015). Asian elephants are not self-sustaining in North America (Fischer, 2011; Wiese, 2000). Therefore, any research that contributes to the breeding success of elephants in captivity (either directly by improving reproduction or indirectly by enhancing behavior and welfare) should be of high interest to collection and population managers.

CHAPTER 4

CONCLUSIONS AND IMPLICATIONS

The concentrations at which chemical signals are emitted have profound effects on the ways that they are perceived. In the present study, Asian elephants of both sexes showed a general increase in chemosensory behavior with increasing concentration; elephants that had and did not have reproductive experience demonstrated this pattern. Perhaps more interesting than this increasing pattern was the effect sex and reproductive experience had on threshold of detection: each compound was first detected at a lower concentration by the opposite sex that produces it naturally, and sexual experience lowered the threshold for each sex for both compounds (except that inexperienced females had a lower threshold for *Z*7-12:Ac than experienced females, who showed no concentration-dependent response). These findings are in direct accordance with the predictions set forth by signaling theory, that animals should respond to signals according to their perceived relevance. Applying these signals to captive elephants in the form of enrichment was more challenging. The bioassay technique used to study Asian elephant pheromones was developed by Rasmussen and her colleagues over thirty years ago. While this method apparently works for studying the intricacies of chemosensory behavior in elephants, it may not be conducive to analyzing the broader behavioral enrichment effects that result from frontalin or *Z*7-12:Ac. At this point in our understanding of chemical signaling in mammals, Asian elephants are unique in that they utilize at least two single-component compounds as pheromones. However, even in captive facilities they are a difficult species to study behaviorally because of their complexity in terms of sociality and intelligence. Our knowledge of how vertebrate

species interact with chemical signals, how olfaction interacts with other sensory modalities, and how animals integrate this information to modulate their behavior is clearly still growing (Schulte et al., 2016).

More broadly, Asian elephants are of particular concern because of their conservation status: they are listed as an endangered species by the International Union for Conservation of Nature (IUCN; Choudhury et al., 2008). As habitat destruction increases in frequency, so does the occurrence of conflict between people and elephants (Baskaran, Kannan, Anbarasan, Thapa, & Sukumar, 2013), leading to the death of hundreds of elephants every year (Gubbi, Swaminath, Poornesha, Bhat, & Raghunath, 2014; Hedges, 2006). Conflict between humans and wildlife is increasingly frequent and economically costly, with damage to crops a pervasive form of this conflict (Woodroffe, Thirdgood, & Rabinowitz, 2005). In much of their natural range, Asian elephants are considered pests due to the extent of their damage to smallholder farms, forming the core of what is considered human-elephant conflict (Gubbi et al., 2014).

A sound understanding about the biology of the pests forms the foundation of a push-pull strategy for integrated pest management (Cook, Kahn, & Pickett, 2007). With push-pull, aversive stimuli, such as natural chemical signals, repel insects from crops and attractive odors lure them into another area. Natural chemicals are appealing signals for push-pull because of their potential for slow, long-lasting release and high specificity without adverse side effects. Natural associations with biologically relevant chemical signals are likely to be better suited for push-pull management because behavioral responses are strengthened by generations of selective pressure. Several recent studies have shown that this approach is effective in modifying the movement of invertebrate

pests around valuable crops (Hassanali, Herren, Khan, Pickett, & Woodcock, 2008; Khan, Midega, Pittchar, Pickett, & Bruce, 2011; Khan, Midega, Wadhams, Pickett, & Mumuni, 2007). Theoretically, a push-pull strategy can be applied to vertebrate pests, although our understanding of chemical communication in vertebrates remains a limitation in using this approach. As such, chemical signals have not been implemented in a push-pull fashion, despite the potential for doing so (Schulte, 2016).

A number of studies have investigated the efficacy of various deterrents on Asian and African elephants. Recently, the most popular mitigation strategies have involved using chili peppers or live bees to encourage elephants to stay away from crops. Capsaicin (an active component in chili peppers) appears to activate nociceptors particularly well to deter crop-raiding elephants, as the trunk tips of elephants are well innervated (Davies et al., 2011; Osborn & Rasmussen, 1995; Rasmussen, 2006; Rasmussen & Munger, 1996). Furthermore, beehives have been attached to fences that agitate the bees when disturbed by elephants (Karidozo & Osborn, 2005; Vollrath & Douglas-Hamilton, 2002). Ndlovu, Devereux, Chieffe, Asklof, and Russo (2016) also found that most African elephants in their study fled from bee noise paired with the scent of honey. It is assumed that most crop-raiding elephants have already formed a naturally fearful association with bees, but the beehive fences are certainly not impermeable, and elephants may habituate to bees or bee sounds (Goodyear & Schulte, 2015), or even successfully thwart beehive barriers (King, Douglas-Hamilton, & Vollrath, 2011; King, Lawrence, Douglas-Hamilton, & Vollrath, 2009). Other strategies to deter elephants have involved visual deterrents, including fire and flashing lights, while others have used sounds—including man-made acoustic noises and biologically relevant recorded sounds

(Perera, 2009; Sitati & Walpole, 2006; Thuppil & Coss, 2013; Wijayagunawardane et al., 2016).

Evidence that illustrates the general effectiveness of these strategies is limited—indeed, some studies have yielded conflicting results [e.g. Hedges and Gunaryadi (2010) and Wiafe and Sam (2014) reached different conclusions on the efficacy of chili pepper fences]—and projects involving these mitigation practices on Asian elephants are deficient. Furthermore, these strategies do not fall under the scope of a push-pull approach because they do not simultaneously deter elephants from one area and attract them to another. There is an overall lack of information regarding the effectiveness of using chemicals like pheromones as deterrents or attractants to develop a push-pull strategy.

Elephants navigate their environments and communicate with conspecifics through a number of different modalities, including through visual, vocal, tactile, and chemical channels (Rasmussen, Gunawardena, & Rasmussen, 1997; Langbauer, 2000). Recently, African elephants were reported to have the largest known repertoire of functional olfactory receptor genes, further validating the importance of chemical communication in particular to the social organization of elephants (Niimura et al., 2014). Due to their status as a scent-oriented species, and because of the two known pheromones they use, Asian elephants are a good target species to implement a chemically based push-pull management strategy. By using biologically relevant signals that they would encounter in a natural realm, the potential for success is high to repel elephants from areas of human interest while simultaneously attracting them towards desired habitats. However, the results of this study indicate that these applications need to be optimized

for specific elephants. Indeed, a better understanding of the behavioral ecology of any species inevitably leads to more holistic (and likely more effective) *in-situ* and *ex-situ* management strategies, and the need to manage certain species can form the motivation to investigate the effects of contextual and environmental factors on behavior (Roitberg, 2007).

Carefully managed captive populations should be considered valuable resources to behavioral ecologists, because they provide unparalleled access to animals with known life histories and it can be easier for researchers to manipulate the environment to test for effects on behavior (e.g. Greenwood & Rasmussen, 2009). It would be virtually impossible to study the effects of signal concentration on the chemosensory behavior of wild elephants to the detail that this study reports. During the process of data collection, researchers should make a responsible effort to contribute knowledge that benefits the sustainability of captive populations, especially when study species are endangered. While I propose the results from this study further our understanding of chemical signaling in the light of signal detection theory, this information is certainly of value for and can be applied to *in-situ* and *ex-situ* management strategies. Despite the dedication of numerous researchers before me, our understanding of chemical communication in Asian elephants (and vertebrates in general) is still young, and I predict advances in chemical ecology that are still to come will inevitably develop our knowledge for the betterment of wild and captive elephants.

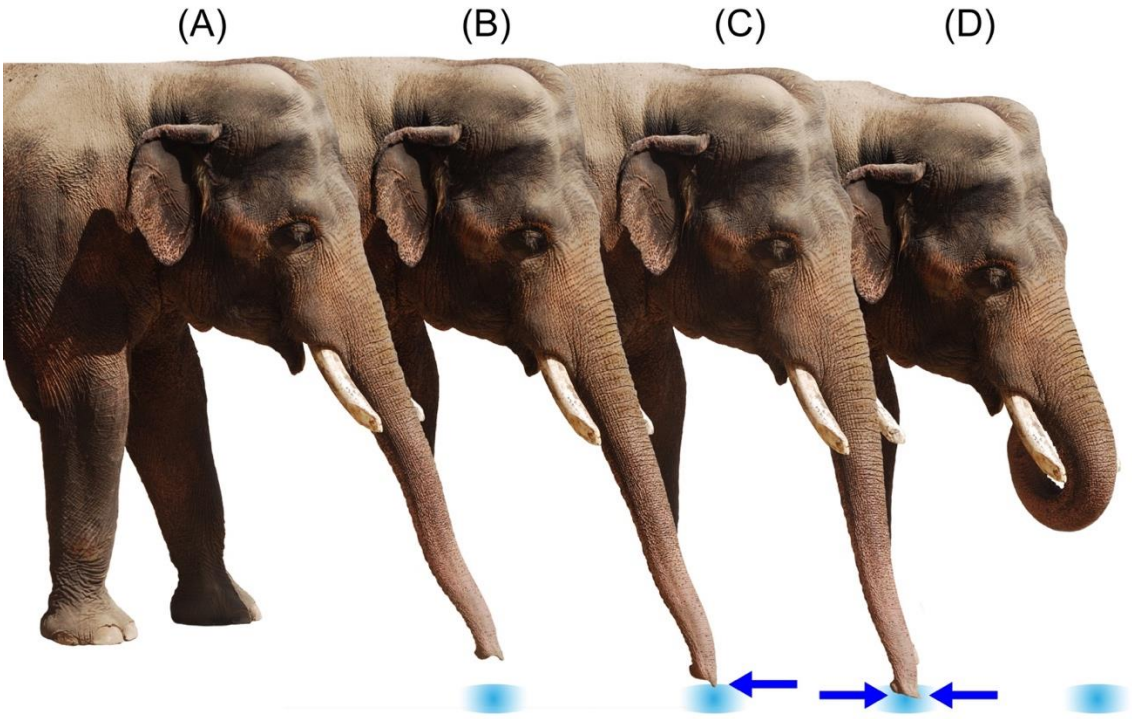

Figure 2.1. Sequential diagram of chemosensory behaviors of interest: sniff (A), check (B), place (C), and flehmen (D). Blue circles indicate a chemical sample, while arrows show areas of contact between trunk and the sample. Photo credit: Chase LaDue.

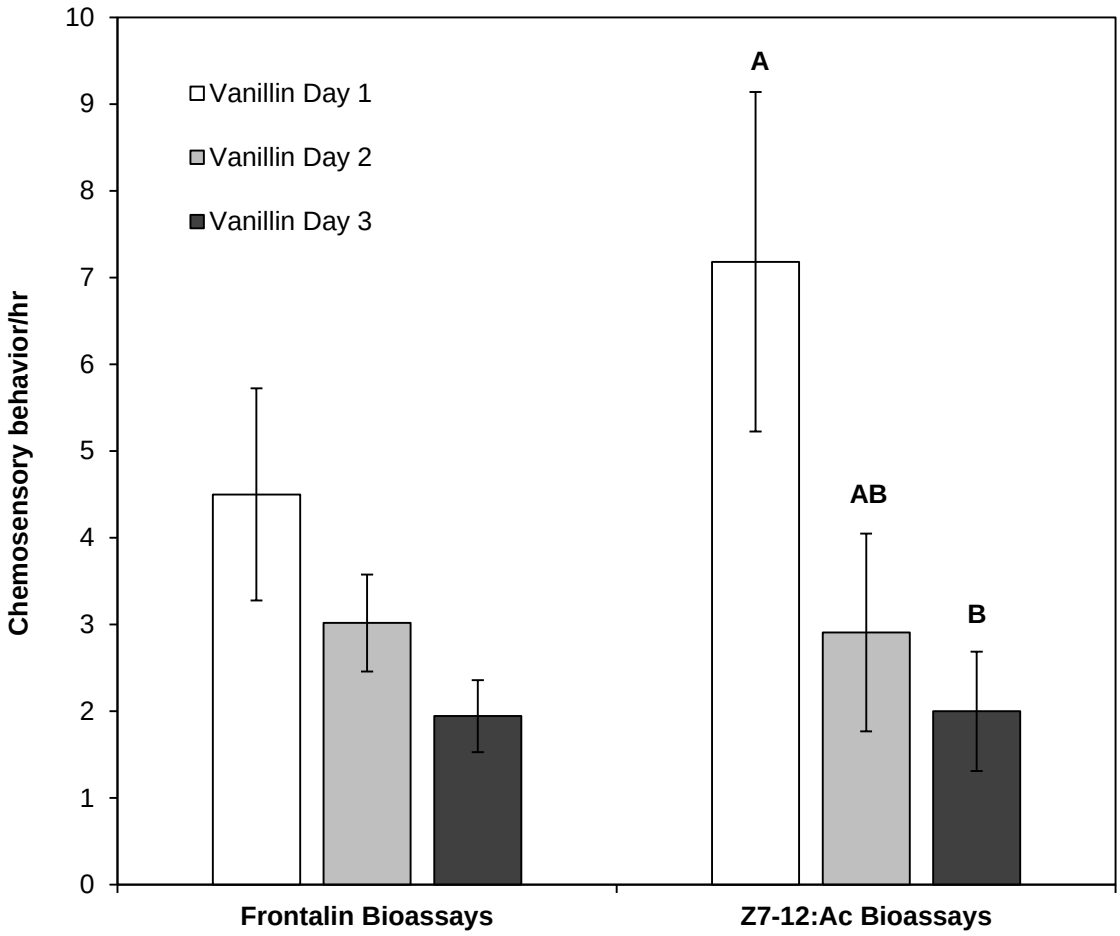

Figure 2.2. Mean ± SE chemosensory behaviors per hour to 1.0 mM vanillin sample on day 1 (white bars), day 2 (light gray bars), and day 3 (dark gray bars) of bioassays for both frontalin and *Z*7-12:Ac sets. For the *Z*7-12:Ac set, different letters denote statistically significant differences in response rates to vanillin across days ($P = 0.031$).

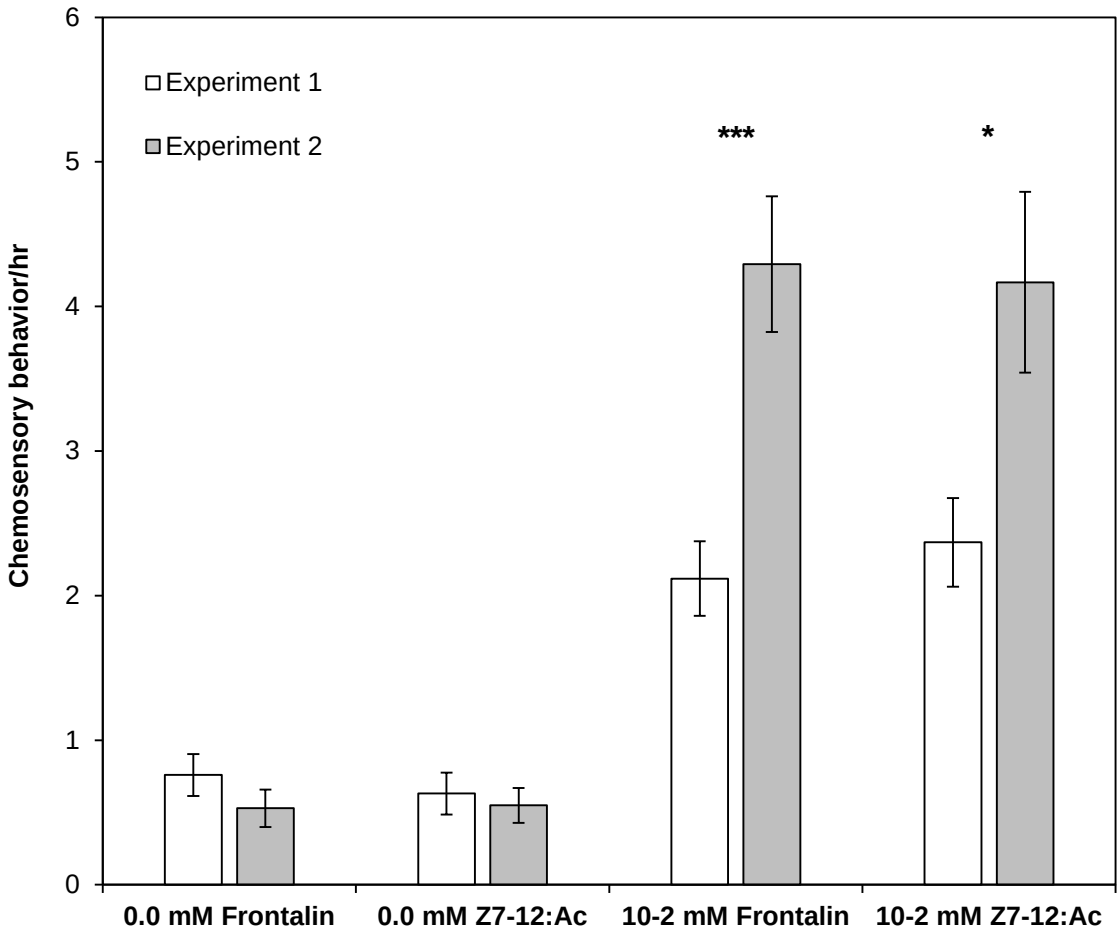

Figure 2.3. Mean ± SE chemosensory behaviors per hour to 0.0 mM and 10^{-2} mM samples of frontalin and *Z*7-12:Ac samples during experiment 1 (three-day bioassays with mid-range concentrations; white bars) and experiment 2 (single-day bioassays with high and low concentrations; gray bars). * denotes $P = 0.006$ between experiments, *** denotes $P < 10^{-4}$.

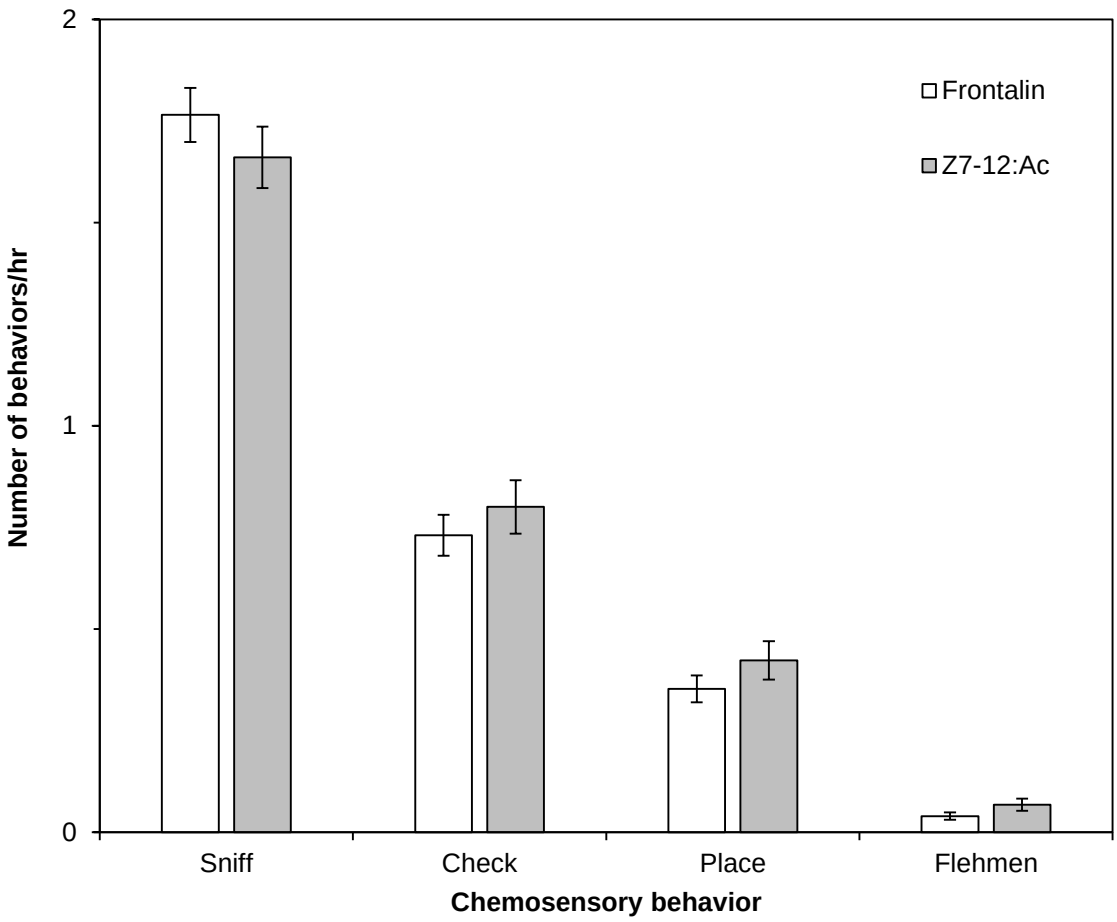

Figure 2.4. Mean ± SE frequencies of chemosensory behaviors for frontalin (white bars) and *Z*7-12:Ac (gray bars) bioassays. Only 10^{-7} mM – 2.0 mM samples are included in analysis (0.0 mM samples have been excluded).

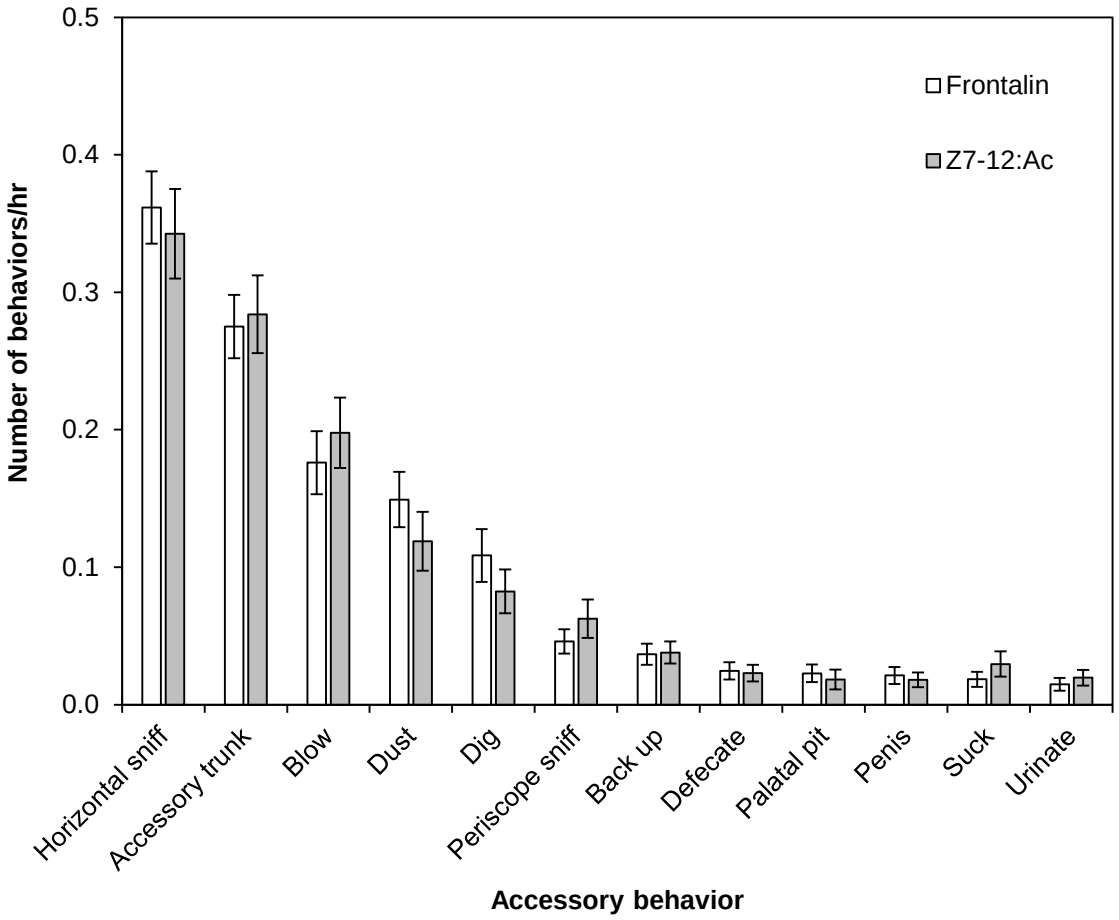

Figure 2.5. Mean ± SE frequencies of accessory behaviors for frontalin (white bars) and *Z*7-12:Ac (gray bars) bioassays. Only 10^{-7} mM – 2.0 mM samples are included in analysis (0.0 mM samples have been excluded). Only accessory behaviors that occurred at an average rate of 0.01 behaviors/hrs are included in this graph.

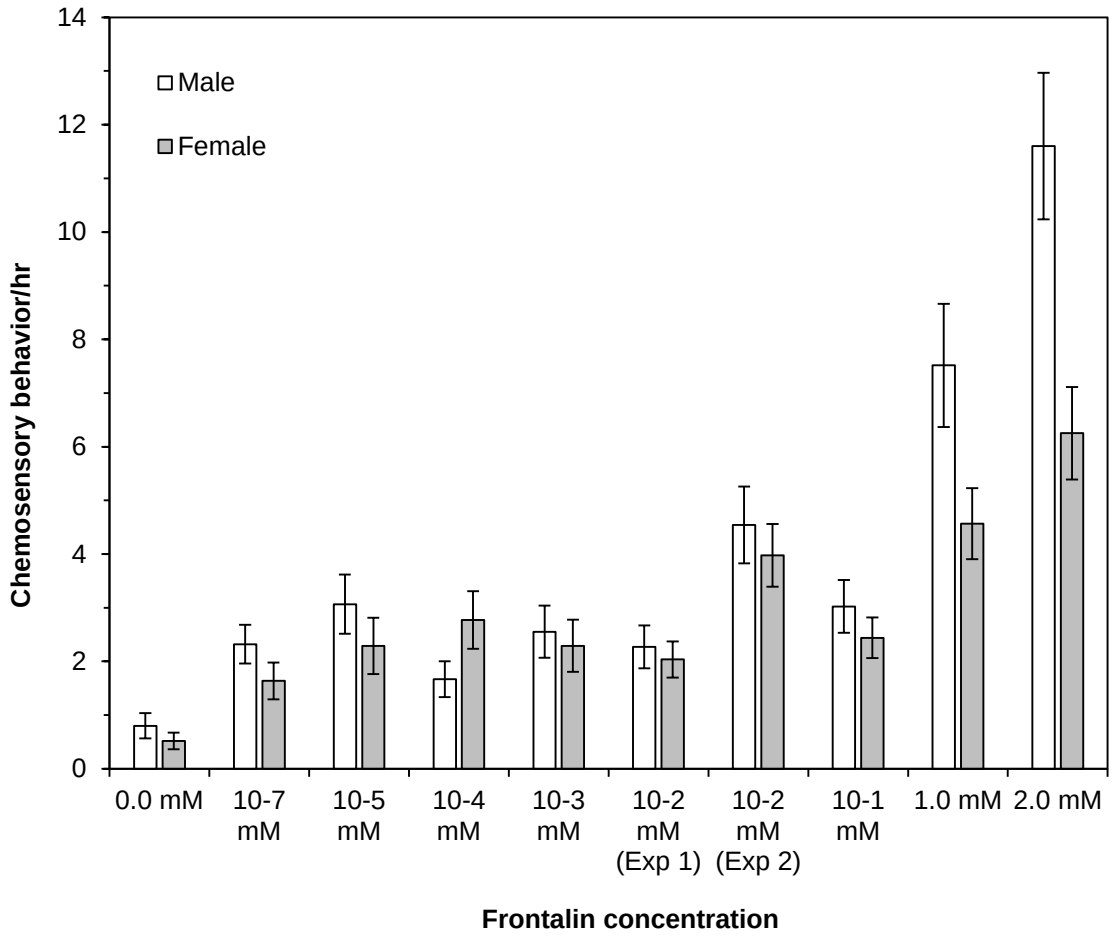

Figure 2.6. Mean ± SE chemosensory behaviors per hour to each frontalin sample, separated by males (white bars) and females (gray bars). 10^{-2} mM samples are divided into responses during the first (Exp 1) and second (Exp 2) experiments.

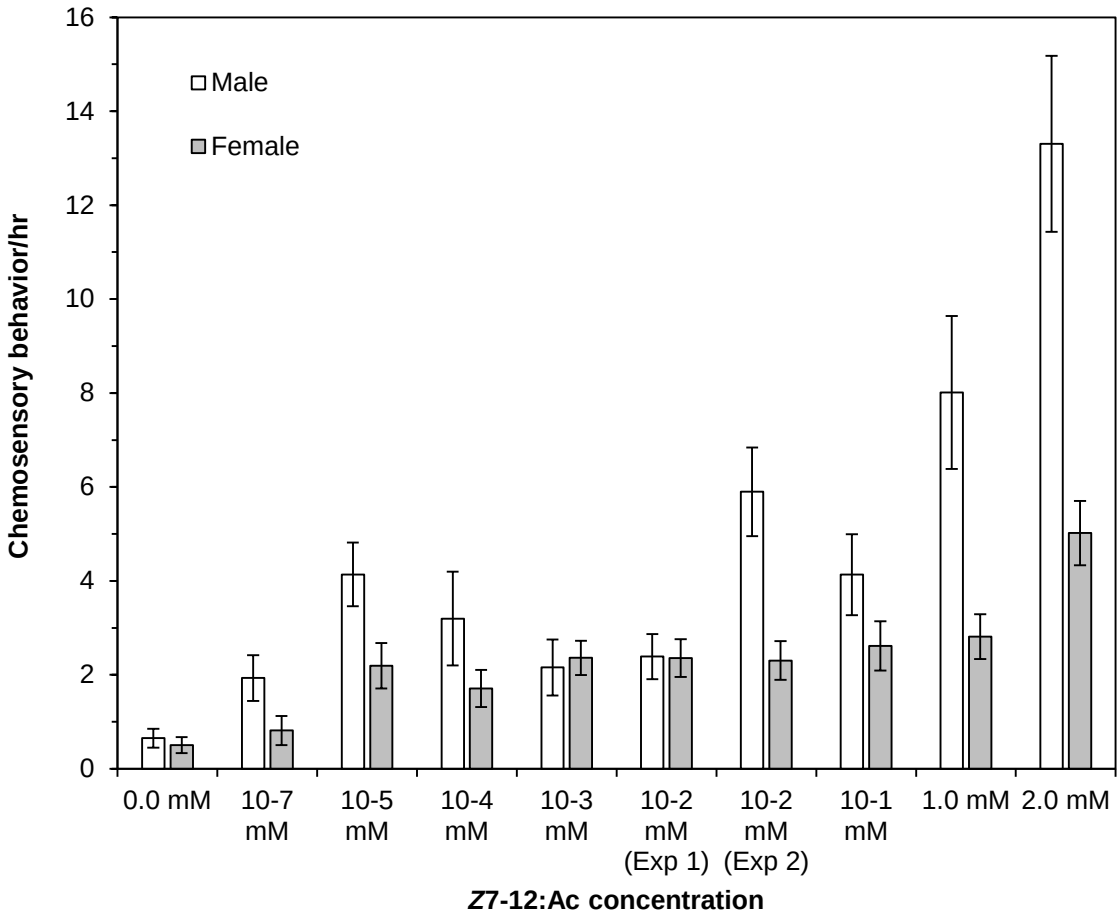

Figure 2.7. Mean ± SE chemosensory behaviors per hour to each *Z*7-12:Ac sample, separated by males (white bars) and females (gray bars). 10^{-2} mM samples are divided into responses during the first (Exp 1) and second (Exp 2) experiments.

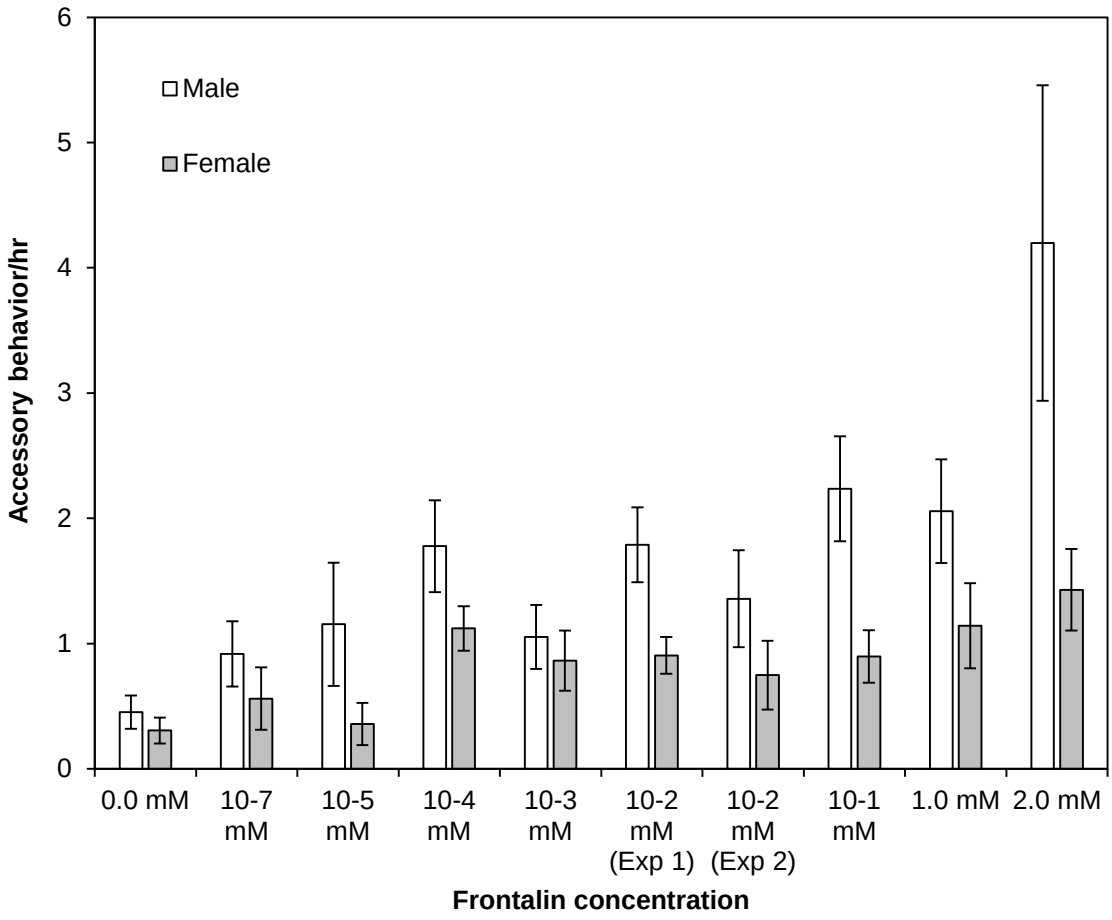

Figure 2.8. Mean ± SE accessory behaviors per hour to each frontalin sample, separated by males (white bars) and females (gray bars). 10^{-2} mM samples are divided into responses during the first (Exp 1) and second (Exp 2) experiments.

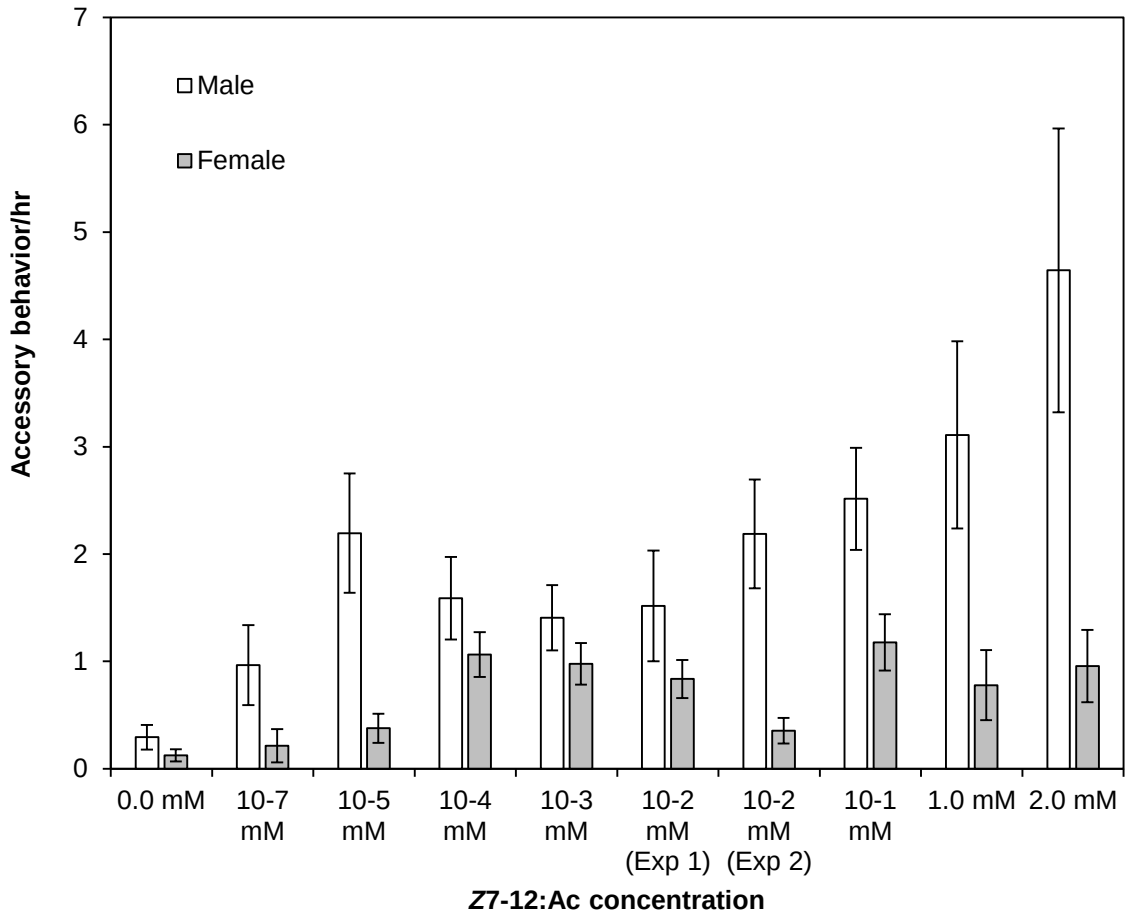

Figure 2.9. Mean ± SE accessory behaviors per hour to each *Z*7-12:Ac sample, separated by males (white bars) and females (gray bars). 10^{-2} mM samples are divided into responses during the first (Exp 1) and second (Exp 2) experiments.

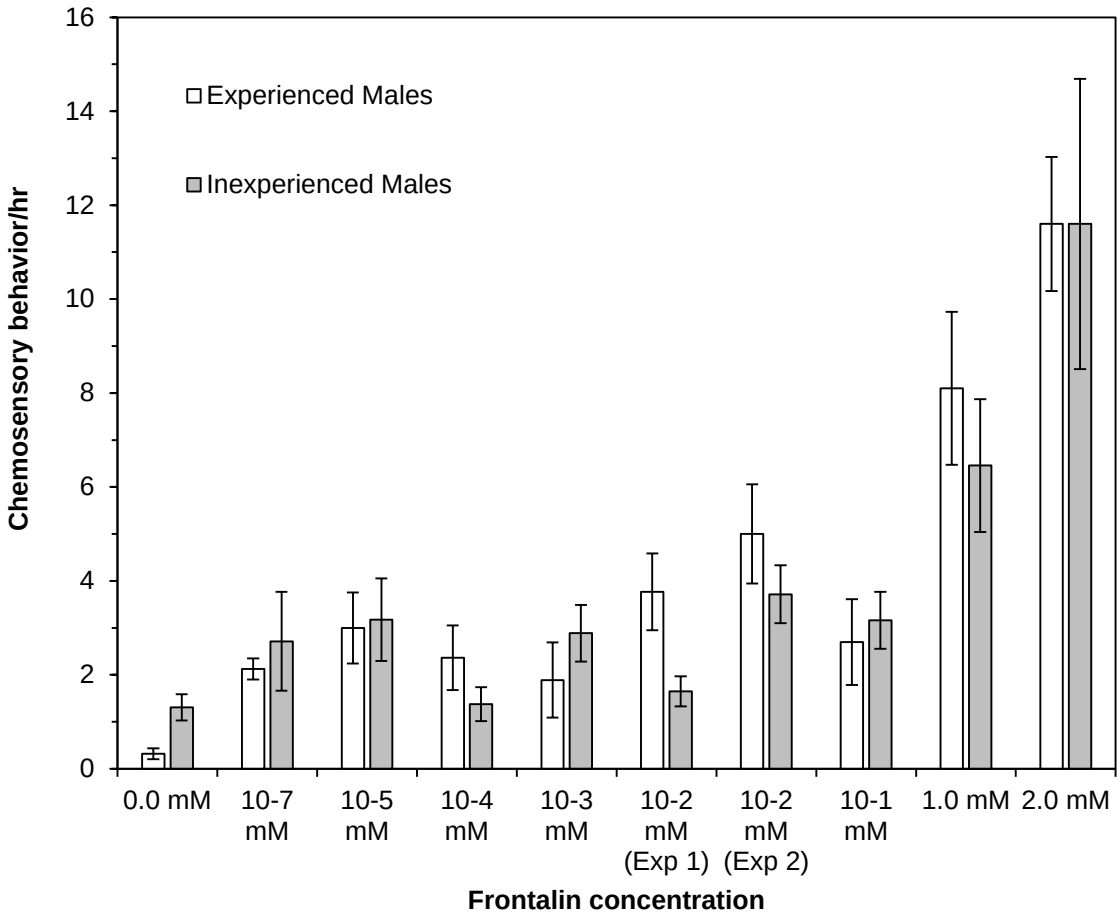

Figure 2.10. Mean ± SE chemosensory behaviors per hour to frontalin samples for reproductively experienced (white bars) and inexperienced (gray bars) males. 10^{-2} mM samples are divided into responses during the first (Exp 1) and second (Exp 2) experiments.

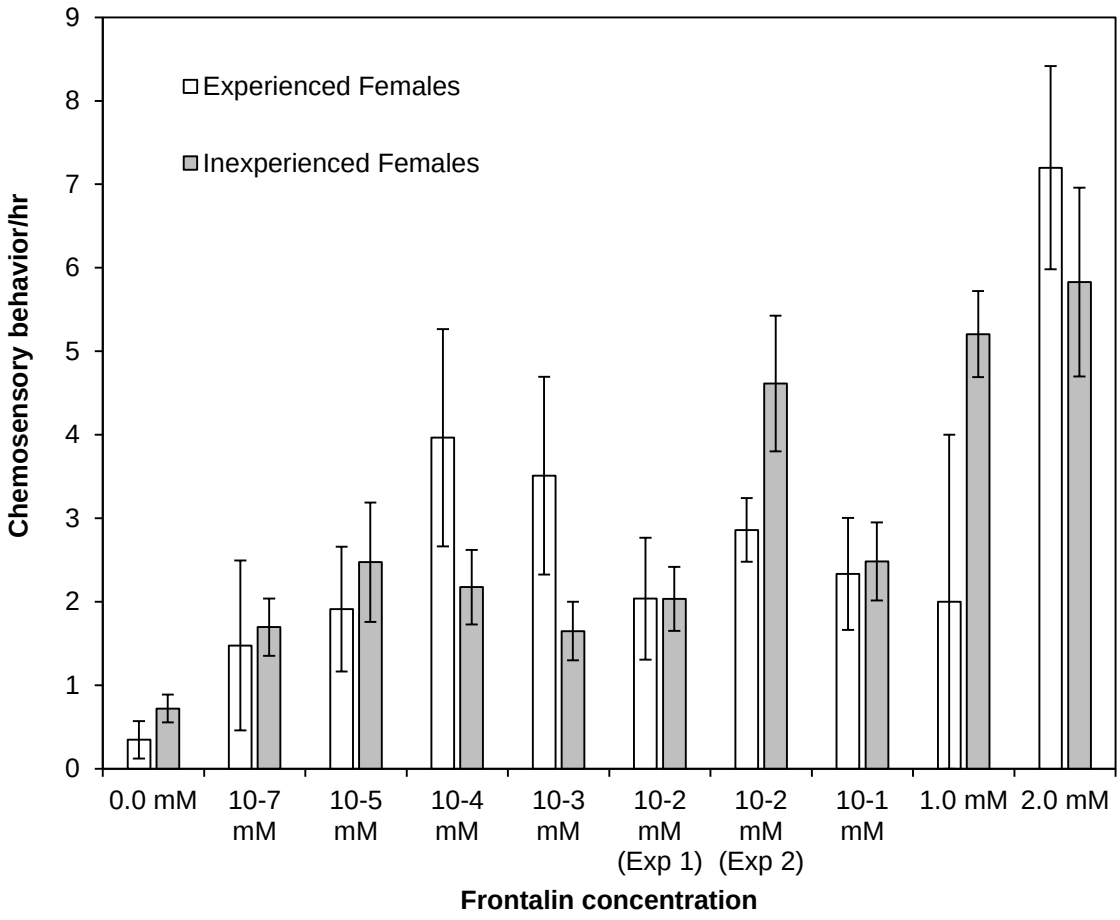

Figure 2.11. Mean ± SE chemosensory behaviors per hour to frontalin samples for reproductively experienced (white bars) and inexperienced (gray bars) females. 10^{-2} mM samples are divided into responses during the first (Exp 1) and second (Exp 2) experiments.

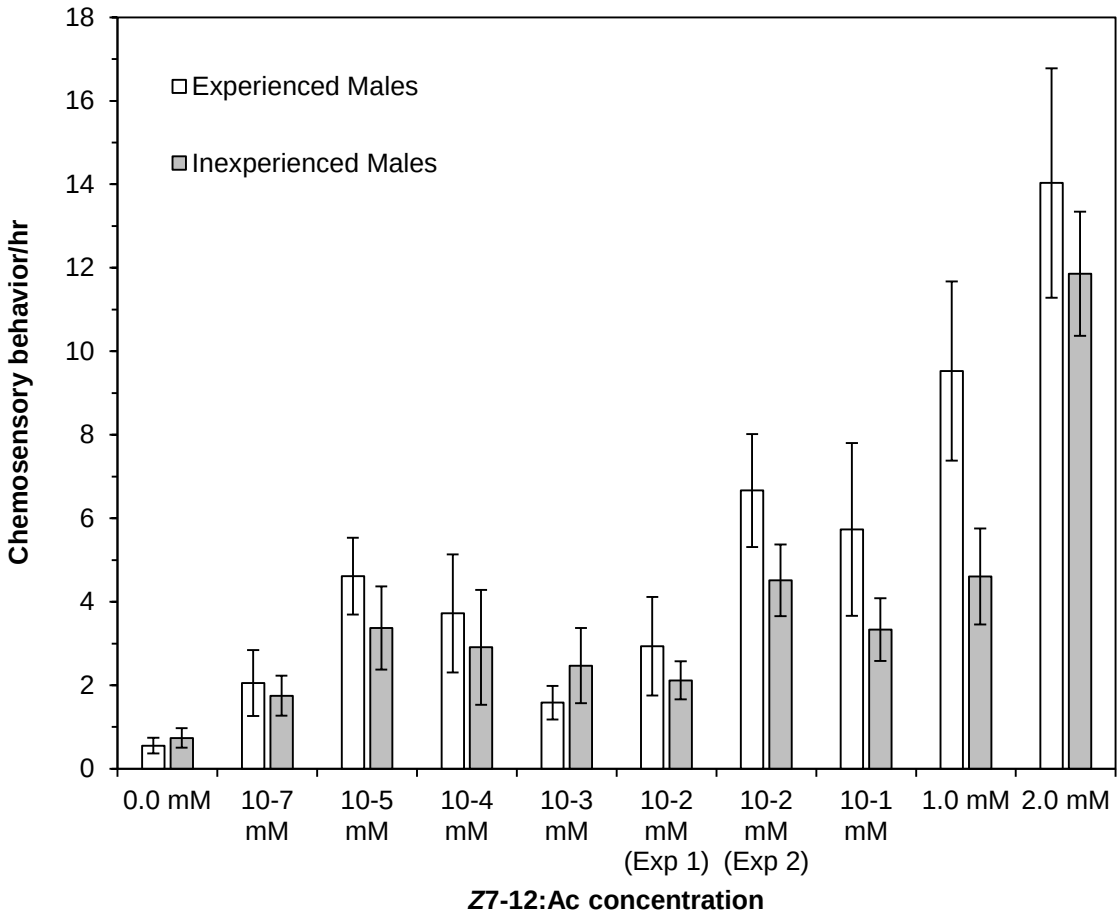

Figure 2.12. Mean ± SE chemosensory behaviors per hour to *Z*7-12:Ac samples for reproductively experienced (white bars) and inexperienced (gray bars) males. 10^{-2} mM samples are divided into responses during the first (Exp 1) and second (Exp 2) experiments.

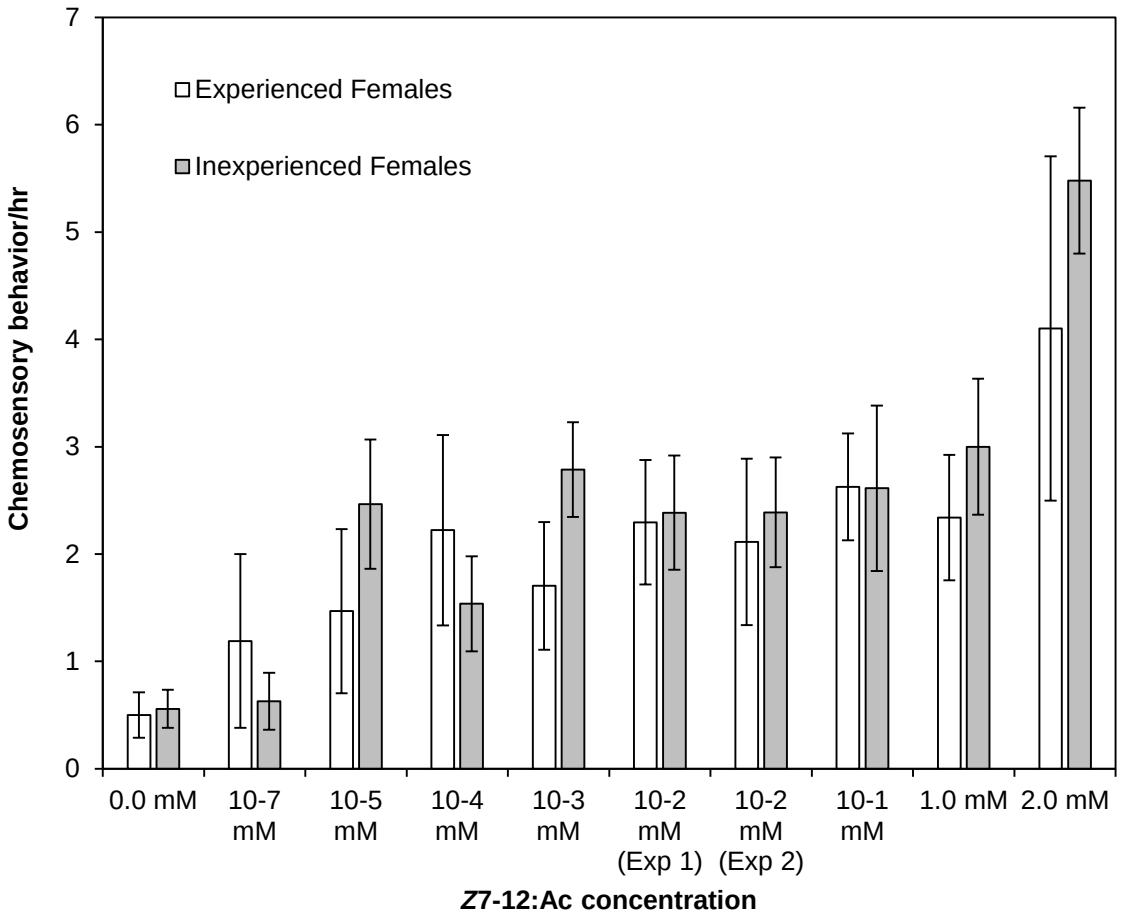

Figure 2.13. Mean ± SE chemosensory behaviors per hour to *Z*7-12:Ac samples for reproductively experienced (white bars) and inexperienced (gray bars) females. 10^{-2} mM samples are divided into responses during the first (Exp 1) and second (Exp 2) experiments.

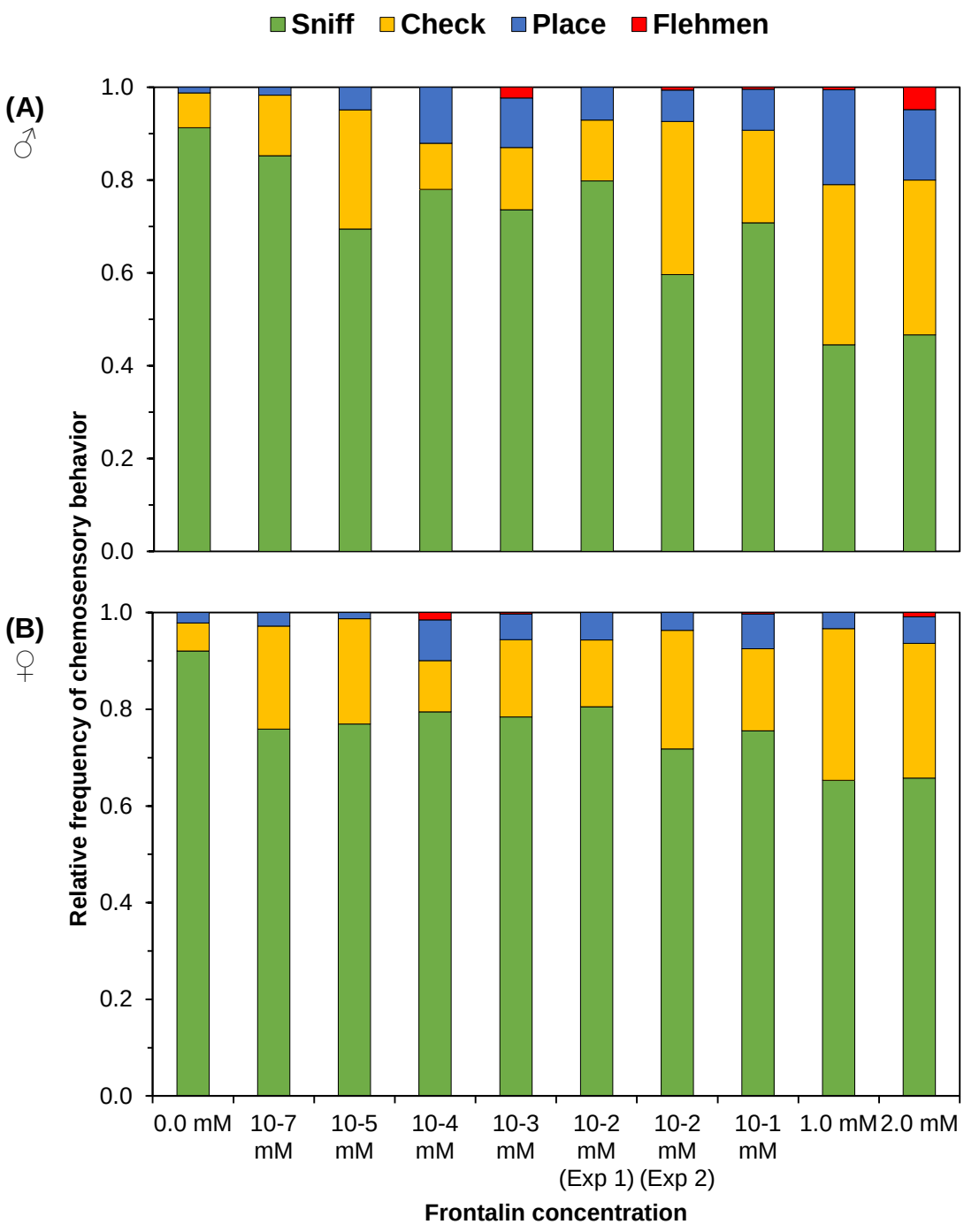

Figure 2.14. Relative frequencies of chemosensory behavior (sniff, check, place, and flehmen) exhibited towards frontalin samples in **(A)** males and **(B)** females. 10^{-2} mM samples are divided into responses during the first (Exp 1) and second (Exp 2) experiments.

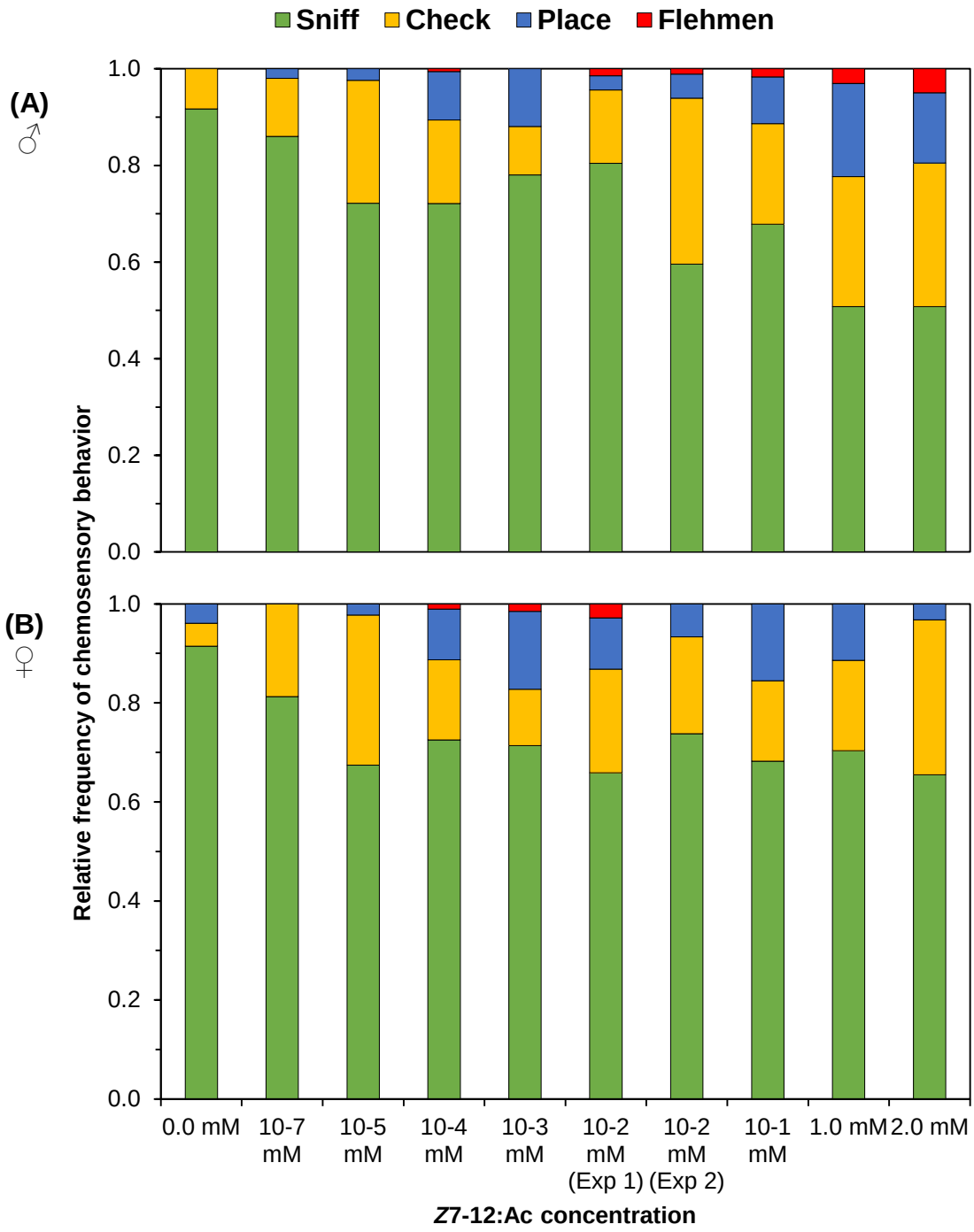

Figure 2.15. Relative frequencies of chemosensory behavior (sniff, check, place, and flehmen) exhibited towards *Z*7-12:Ac samples in **(A)** males and **(B)** females. 10^{-2} mM samples are divided into responses during the first (Exp 1) and second (Exp 2) experiments.

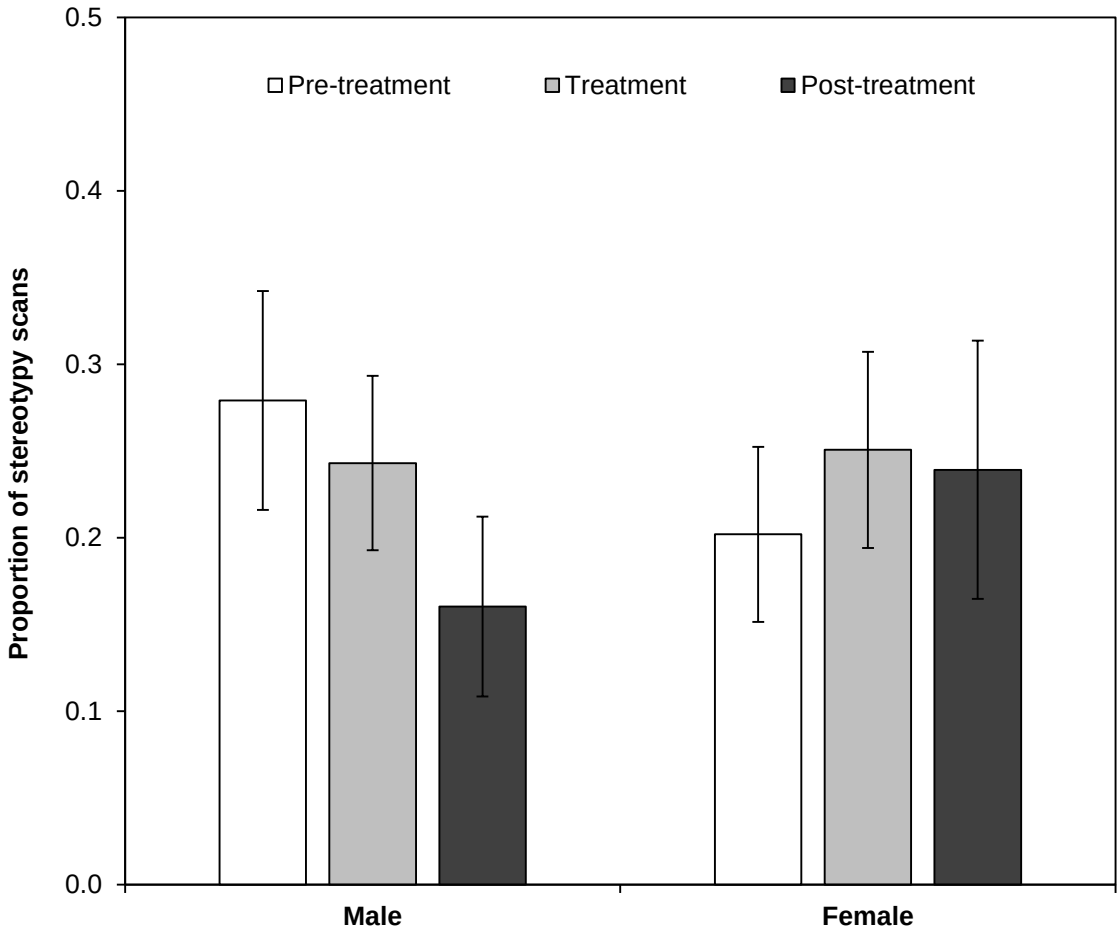

Figure 3.1. Mean ± SE proportion of scans spent engaged in stereotypy for males and females, the day before samples were placed (pre-treatment, white bars), during bioassays when samples are present (treatment, light gray bars) and the day after samples were removed (post-treatment, dark gray bars). Frontalin and *Z*7-12:Ac are combined here. Only elephants that exhibited stereotypic behavior during pre-treatment are included here.

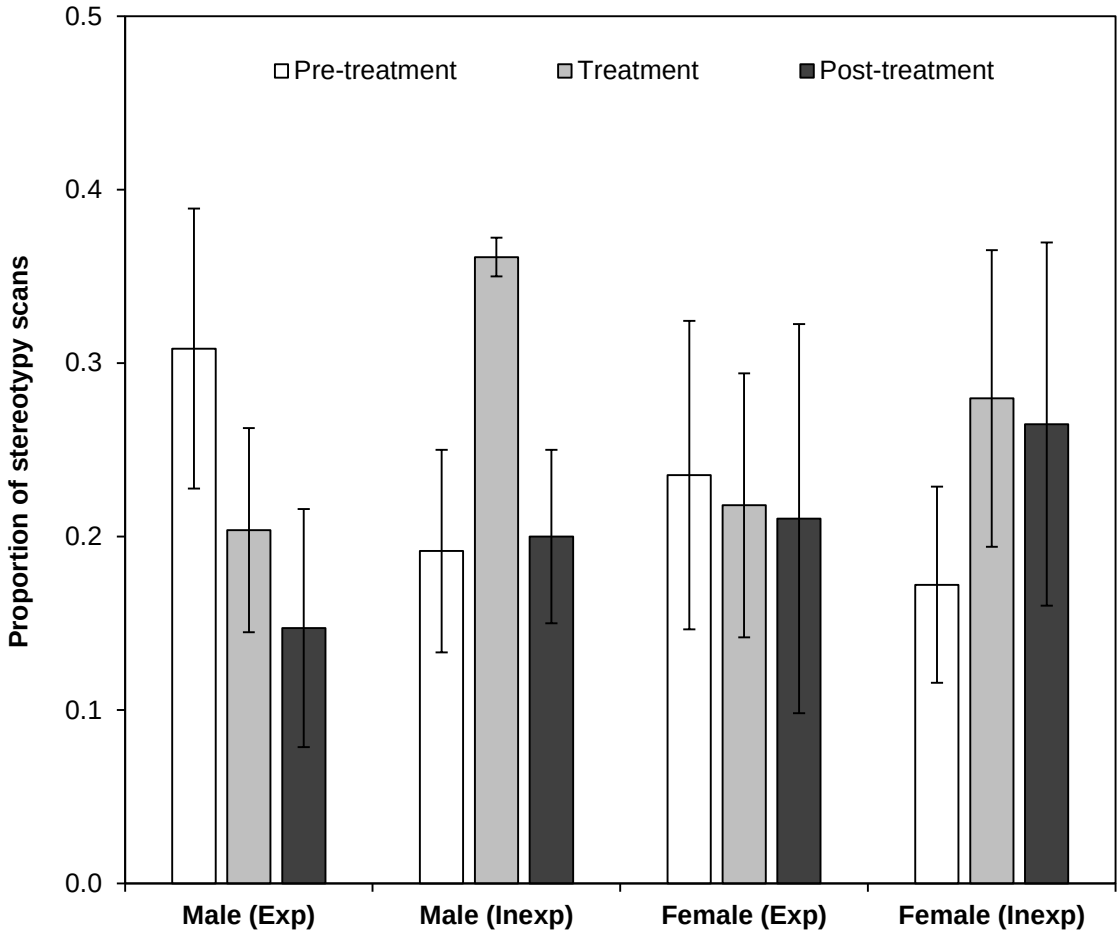

Figure 3.2. Mean ± SE proportion of scans spent engaged in stereotypy for males and females that were reproductively experienced (Exp) and inexperienced (Inexp), the day before samples were placed (pre-treatment, white bars), during bioassays when samples were present (treatment, light gray bars), and the day after samples were removed (post-treatment, dark gray bars). Frontalin and *Z*7-12:Ac are combined here. Only elephants that exhibited stereotypic behavior during pre-treatment are included here.

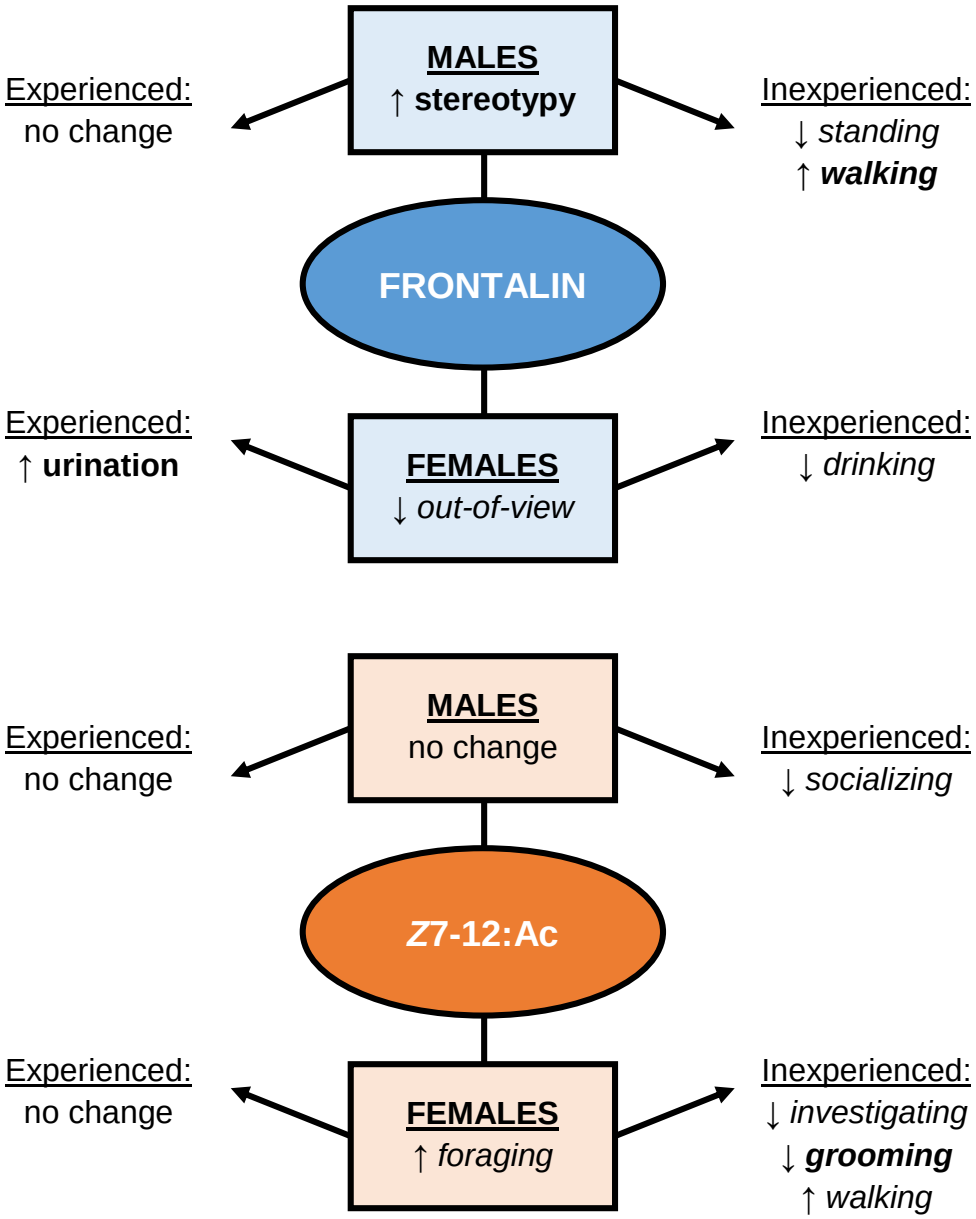

Figure 3.3. Flow chart summarizing the behavioral effects of frontalin and *Z*7-12:Ac. Arrows represent an increase (↑) or decrease (↓) in a particular behavior. Bolded behaviors changed significantly ($P < 0.05$) when samples were present (treatment phase) compared to pre-treatment phase, and italicized behaviors changed significantly during post-treatment phase compared to pre-treatment or treatment. Behaviors that are bolded and italicized are those that differ significantly from pre-treatment phase. Behaviors in male and female boxes indicate significant changes when reproductive experience categories are analyzed together.

TABLES

Table 2.1. Outline of experiment 1, indicating the order with which *Z*7-12:Ac and frontalin sets were presented during a typical site visit. Each set consisted of five concentration samples (0.0 mM – 10^{-1} mM) plus a 1.0 mM vanillin sample. At 9 of 10 facilities, the elephants were separated into two groups (the tenth facility had too many elephants to assay in only two groups, but the following pattern was expanded to accommodate three groups to be observed each day). The labels 'Group A' and 'Group B' were assigned based on the order of observation. The order of presentation for each compound set across both groups was alternated between visits to each facility (i.e. at the facility after this hypothetical site, Group A would receive the frontalin set in AM, and Group B would receive the *Z*7-12:Ac set in AM).

		Group A	**Group B**
Day 1	AM	*no samples present*	*no samples present*
	PM	*no samples present*	*no samples present*
Day 2	AM	*Z*7-12:Ac	Frontalin
	PM	Frontalin	*Z*7-12:Ac
Day 3	AM	*Z*7-12:Ac	Frontalin
	PM	Frontalin	*Z*7-12:Ac
Day 4	AM	*Z*7-12:Ac	Frontalin
	PM	Frontalin	*Z*7-12:Ac
Day 5	AM	*no samples present*	*no samples present*
	PM	*no samples present*	*no samples present*

Table 2.2. Ethogram of chemosensory (Chemo.) and accessory (Access.) behaviors used during bioassays. Behaviors and descriptions modified from Schulte and Rasmussen (1999) and Bagley et al. (2006).

	Behavior	Description
	Proximity	Elephant within 1 body length of sample
	Near	Elephant within 1 trunk length of sample
Chemo.	Sniff	Trunk tip held over sample or directed toward samples
	Check	Placement of trunk tip finger into sample
	Place	Using entire end of trunk (flattened) on sample
	Flehmen	Placement of trunk tip onto orifice of vomeronasal ducts
Access.	Accessory trunk	Includes trunk flick (bottom of trunk), pinch, and wriggle (slower than flick, including most of trunk in movement)
	Aborted flehmen	Flehmen-like trunk curl without contact to vomeronasal ducts
	Blow	Forceful exhalation
	Back up	Reverse or backwards directional change
	Defecate	Voiding feces
	Dig	Using trunk tip or foot to displace ground at sample area
	Dust	Using trunk to throw sample (in sand, dirt, etc.) over body
	Ear flap	Regular movements of ear(s) (>1 every 3 sec)
	Head shake	Head quivers
	Horizontal tail	Tail erect
	Horizontal sniff	Raises trunk above ground (but not above head) in an apparent attempt to smell air
	Mouth blow	Blow in mouth with trunk
	Penis	Penis dropping (without urination), belly hitting, penis pull, or other male behavior involving penis
	Palatal pit	Trunk tip contact onto the palatal pit area in a sideways motion
	Periscope sniff	Raises trunk above head level and holds this position for at least 2 sec
	Suck	Trunk tip in sample, muscle contraction, and audible noise
	Temporal gland secretion	Streaming from temporal gland(s) on sides of head
	Urinate	Voiding urine
	Vocalize	Audible growl, rumble, trumpet, roar, or squeak
	Other	Other trunk behavior directed towards sample (e.g. pinch, eat substrate, trunk curl)

Table 2.3. Frontalin bioassays: Tukey's HSD pairwise comparisons for **(A)** male and **(B)** female chemosensory responses across all concentrations. Significant differences between concentrations are bolded ($P < 0.050$).

(A) Male	0.0 mM	10^{-7} mM	10^{-5} mM	10^{-4} mM	10^{-3} mM	10^{-2} mM (Exp1)	10^{-2} mM (Exp2)	10^{-1} mM	1.0 mM
10^{-7} mM	0.781								
10^{-5} mM	0.206	0.999							
10^{-4} mM	0.981	1.000	0.917						
10^{-3} mM	0.412	1.000	1.000	0.993					
10^{-2} mM (Exp1)	0.307	1.000	1.000	0.983	1.000				
10^{-2} mM (Exp2)	**0.003**	0.599	0.950	0.157	0.674	0.728			
10^{-1} mM	0.125	0.999	1.000	0.878	1.000	1.000	0.939		
1.0 mM	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	0.068	**<0.001**	
2.0 mM	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**0.002**

(B) Female	0.0 mM	10^{-7} mM	10^{-5} mM	10^{-4} mM	10^{-3} mM	10^{-2} mM (Exp1)	10^{-2} mM (Exp2)	10^{-1} mM	1.0 mM
10^{-7} mM	0.908								
10^{-5} mM	0.313	0.999							
10^{-4} mM	**0.001**	0.887	1.000						
10^{-3} mM	**0.038**	0.997	1.000	0.997					
10^{-2} mM (Exp1)	0.118	1.000	1.000	0.939	1.000				
10^{-2} mM (Exp2)	**<0.001**	0.238	0.671	0.846	0.439	0.224			
10^{-1} mM	**0.014**	0.987	1.000	1.000	1.000	0.999	0.569		
1.0 mM	**<0.001**	0.059	0.277	0.392	0.110	**0.040**	1.000	0.169	
2.0 mM	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	0.222	**<0.001**	0.683

Table 2.4. *Z*7-12:Ac bioassays: Tukey's HSD pairwise comparisons for **(A)** male and **(B)** female chemosensory responses across all concentrations. Significant differences between concentrations are bolded ($P < 0.050$).

(A) Male	0.0 mM	10^{-7} mM	10^{-5} mM	10^{-4} mM	10^{-3} mM	10^{-2} mM (Exp1)	10^{-2} mM (Exp2)	10^{-1} mM	1.0 mM
10^{-7} mM	0.091								
10^{-5} mM	**0.048**	0.885							
10^{-4} mM	**0.032**	0.996	0.999						
10^{-3} mM	**0.009**	1.000	0.869	0.997					
10^{-2} mM (Exp1)	0.066	1.000	0.986	1.000	1.000				
10^{-2} mM (Exp2)	**<0.001**	0.176	0.948	0.517	0.111	0.300			
10^{-1} mM	**0.047**	0.866	1.000	0.999	0.841	0.982	0.936		
1.0 mM	**<0.001**	**0.002**	0.129	**0.009**	**<0.001**	**0.002**	0.899	0.100	
2.0 mM	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**0.007**

(B) Female	0.0 mM	10^{-7} mM	10^{-5} mM	10^{-4} mM	10^{-3} mM	10^{-2} mM (Exp1)	10^{-2} mM (Exp2)	10^{-1} mM	1.0 mM
10^{-7} mM	1.000								
10^{-5} mM	0.241	0.726							
10^{-4} mM	0.372	0.929	0.999						
10^{-3} mM	**0.014**	0.339	1.000	0.966					
10^{-2} mM (Exp1)	**0.015**	0.383	1.000	0.983	1.000				
10^{-2} mM (Exp2)	**0.043**	0.395	1.000	0.967	1.000	1.000			
10^{-1} mM	**0.002**	0.151	1.000	0.787	1.000	1.000	1.000		
1.0 mM	**0.006**	0.149	0.998	0.733	0.999	0.997	1.000	1.000	
2.0 mM	**<0.001**	**<0.001**	**0.009**	**<0.001**	**0.002**	**<0.001**	**0.016**	**0.010**	0.069

Table 2.5. Frontalin bioassays: Tukey's HSD pairwise comparisons for **(A)** male and **(B)** female accessory responses across all concentrations. Significant differences between concentrations are bolded ($P < 0.050$).

(A) Male	0.0 mM	10^{-7} mM	10^{-5} mM	10^{-4} mM	10^{-3} mM	10^{-2} mM (Exp1)	10^{-2} mM (Exp2)	10^{-1} mM	1.0 mM
10^{-7} mM	1.000								
10^{-5} mM	0.991	1.000							
10^{-4} mM	0.641	0.989	0.999						
10^{-3} mM	0.999	1.000	1.000	0.979					
10^{-2} mM (Exp1)	0.591	0.984	0.999	1.000	0.970				
10^{-2} mM (Exp2)	0.940	1.000	1.000	1.000	1.000	1.000			
10^{-1} mM	0.381	0.938	0.989	1.000	0.891	1.000	0.999		
1.0 mM	0.395	0.926	0.984	1.000	0.879	1.000	0.998	1.000	
2.0 mM	**<0.001**	**0.001**	**0.003**	**0.016**	**<0.001**	**0.020**	**0.007**	**0.046**	0.090

(B) Female	0.0 mM	10^{-7} mM	10^{-5} mM	10^{-4} mM	10^{-3} mM	10^{-2} mM (Exp1)	10^{-2} mM (Exp2)	10^{-1} mM	1.0 mM
10^{-7} mM	1.000								
10^{-5} mM	1.000	1.000							
10^{-4} mM	0.437	0.986	0.857						
10^{-3} mM	**0.036**	0.977	0.810	1.000					
10^{-2} mM (Exp1)	0.824	1.000	0.979	1.000	0.999				
10^{-2} mM (Exp2)	**0.012**	0.999	0.980	1.000	1.000	1.000			
10^{-1} mM	0.694	0.998	0.950	1.000	1.000	1.000	1.000		
1.0 mM	**0.046**	0.955	0.798	1.000	1.000	0.995	1.000	0.999	
2.0 mM	**0.047**	**0.016**	**0.024**	0.901	0.938	0.636	0.941	0.767	0.999

Table 2.6. *Z*7-12:Ac bioassays: Tukey's HSD pairwise comparisons for **(A)** male and **(B)** female accessory responses across all concentrations. Significant differences between concentrations are bolded ($P < 0.050$).

(A) Male	0.0 mM	10^{-7} mM	10^{-5} mM	10^{-4} mM	10^{-3} mM	10^{-2} mM (Exp1)	10^{-2} mM (Exp2)	10^{-1} mM	1.0 mM
10^{-7} mM	0.996								
10^{-5} mM	0.243	0.959							
10^{-4} mM	0.815	1.000	0.994						
10^{-3} mM	0.830	1.000	0.993	1.000					
10^{-2} mM (Exp1)	0.952	1.000	0.974	1.000	1.000				
10^{-2} mM (Exp2)	0.217	0.957	1.000	0.994	0.992	0.972			
10^{-1} mM	0.074	0.844	1.000	0.942	0.935	0.864	1.000		
1.0 mM	**0.004**	0.322	0.965	0.412	0.396	0.297	0.959	0.994	
2.0 mM	**<0.001**	**0.003**	0.089	**0.002**	**0.002**	**0.001**	0.076	0.151	0.740

(B) Female	0.0 mM	10^{-7} mM	10^{-5} mM	10^{-4} mM	10^{-3} mM	10^{-2} mM (Exp1)	10^{-2} mM (Exp2)	10^{-1} mM	1.0 mM
10^{-7} mM	1.000								
10^{-5} mM	1.000	1.000							
10^{-4} mM	0.051	0.490	0.701						
10^{-3} mM	0.202	0.771	0.910	1.000					
10^{-2} mM (Exp1)	0.458	0.943	0.988	0.986	1.000				
10^{-2} mM (Exp2)	1.000	1.000	1.000	0.598	0.858	0.977			
10^{-1} mM	**0.001**	0.073	0.166	0.981	0.844	0.434	0.103		
1.0 mM	0.490	0.901	0.968	1.000	1.000	1.000	0.949	0.885	
2.0 mM	0.211	0.660	0.816	1.000	1.000	0.996	0.753	0.997	1.000

Table 2.7. Frontalin bioassays: Tukey's HSD pairwise comparisons for **(A)** reproductively experienced (Exp.) and **(B)** inexperienced (Inexp.) male chemosensory responses across all concentrations. Significant differences between concentrations are bolded ($P < 0.050$).

(A) Exp.	0.0 mM	10^{-7} mM	10^{-5} mM	10^{-4} mM	10^{-3} mM	10^{-2} mM (Exp1)	10^{-2} mM (Exp2)	10^{-1} mM	1.0 mM
10^{-7} mM	0.928								
10^{-5} mM	0.565	1.000							
10^{-4} mM	0.937	1.000	1.000						
10^{-3} mM	0.983	1.000	1.000	1.000					
10^{-2} mM (Exp1)	0.418	0.990	1.000	0.999	0.983				
10^{-2} mM (Exp2)	**0.015**	0.531	0.901	0.802	0.534	0.999			
10^{-1} mM	0.856	1.000	1.000	1.000	1.000	1.000	0.900		
1.0 mM	**<0.001**	**0.002**	**0.014**	**0.017**	**0.003**	0.170	0.377	**0.031**	
2.0 mM	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	0.220

(B) Inexp.	0.0 mM	10^{-7} mM	10^{-5} mM	10^{-4} mM	10^{-3} mM	10^{-2} mM (Exp1)	10^{-2} mM (Exp2)	10^{-1} mM	1.0 mM
10^{-7} mM	0.986								
10^{-5} mM	0.872	1.000							
10^{-4} mM	1.000	0.992	0.906						
10^{-3} mM	0.778	1.000	1.000	0.848					
10^{-2} mM (Exp1)	1.000	0.998	0.965	1.000	0.949				
10^{-2} mM (Exp2)	0.610	1.000	1.000	0.674	1.000	0.807			
10^{-1} mM	0.585	1.000	1.000	0.682	1.000	0.847	1.000		
1.0 mM	**0.002**	0.339	0.441	**0.004**	0.126	**0.008**	0.690	0.205	
2.0 mM	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**0.025**

Table 2.8. Frontalin bioassays: Tukey's HSD pairwise comparisons for **(A)** reproductively experienced (Exp.) and **(B)** inexperienced (Inexp.) female chemosensory responses across all concentrations. Significant differences between concentrations are bolded ($P < 0.050$).

(A) Exp.	0.0 mM	10^{-7} mM	10^{-5} mM	10^{-4} mM	10^{-3} mM	10^{-2} mM (Exp1)	10^{-2} mM (Exp2)	10^{-1} mM	1.0 mM
10^{-7} mM	1.000								
10^{-5} mM	0.987	1.000							
10^{-4} mM	**0.041**	0.904	0.941						
10^{-3} mM	0.118	0.971	0.988	1.000					
10^{-2} mM (Exp1)	0.878	1.000	1.000	0.836	0.964				
10^{-2} mM (Exp2)	0.789	1.000	1.000	1.000	1.000	1.000			
10^{-1} mM	0.740	1.000	1.000	0.933	0.992	1.000	1.000		
1.0 mM	0.998	1.000	1.000	0.993	0.999	1.000	1.000	1.000	
2.0 mM	**<0.001**	0.135	0.136	0.534	0.346	**0.048**	0.369	0.078	0.400

(B) Inexp.	0.0 mM	10^{-7} mM	10^{-5} mM	10^{-4} mM	10^{-3} mM	10^{-2} mM (Exp1)	10^{-2} mM (Exp2)	10^{-1} mM	1.0 mM
10^{-7} mM	0.957								
10^{-5} mM	0.396	0.998							
10^{-4} mM	0.247	1.000	1.000						
10^{-3} mM	0.840	1.000	0.988	0.997					
10^{-2} mM (Exp1)	0.333	1.000	1.000	1.000	1.000				
10^{-2} mM (Exp2)	**<0.001**	0.082	0.444	0.092	**0.015**	**0.050**			
10^{-1} mM	0.061	0.990	1.000	1.000	0.919	0.998	0.215		
1.0 mM	**<0.001**	**0.009**	0.103	**0.006**	**<0.001**	**0.002**	1.000	**0.019**	
2.0 mM	**<0.001**	**<0.001**	**0.010**	**<0.001**	**<0.001**	**<0.001**	0.952	**<0.001**	1.000

Table 2.9. *Z*7-12:Ac bioassays: Tukey's HSD pairwise comparisons for **(A)** reproductively experienced (Exp.) and **(B)** inexperienced (Inexp.) male chemosensory responses across all concentrations. Significant differences between concentrations are bolded ($P < 0.050$).

(A) Exp.	0.0 mM	10^{-7} mM	10^{-5} mM	10^{-4} mM	10^{-3} mM	10^{-2} mM (Exp1)	10^{-2} mM (Exp2)	10^{-1} mM	1.0 mM
10^{-7} mM	**0.049**								
10^{-5} mM	**0.045**	0.980							
10^{-4} mM	0.089	1.000	1.000						
10^{-3} mM	0.499	1.000	0.942	0.997					
10^{-2} mM (Exp1)	0.099	1.000	0.999	1.000	1.000				
10^{-2} mM (Exp2)	**0.048**	0.549	0.991	0.944	0.410	0.849			
10^{-1} mM	**0.040**	0.909	1.000	0.999	0.830	0.988	1.000		
1.0 mM	**<0.001**	**0.039**	0.343	0.233	**0.022**	0.157	0.909	0.836	
2.0 mM	**<0.001**	**<0.001**	**0.001**	**0.001**	**<0.001**	**<0.001**	**0.021**	**0.031**	0.467

(B) Inexp.	0.0 mM	10^{-7} mM	10^{-5} mM	10^{-4} mM	10^{-3} mM	10^{-2} mM (Exp1)	10^{-2} mM (Exp2)	10^{-1} mM	1.0 mM
10^{-7} mM	1.000								
10^{-5} mM	**0.042**	0.995							
10^{-4} mM	0.521	0.999	1.000						
10^{-3} mM	0.079	1.000	1.000	1.000					
10^{-2} mM (Exp1)	0.648	1.000	0.996	0.999	1.000				
10^{-2} mM (Exp2)	**0.015**	0.845	0.999	0.977	0.899	0.793			
10^{-1} mM	**0.031**	0.989	1.000	1.000	0.999	0.987	0.998		
1.0 mM	**0.021**	0.859	0.999	0.980	0.917	0.828	1.000	0.998	
2.0 mM	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**0.003**	**<0.001**	**0.006**

Table 2.10. *Z*7-12:Ac bioassays: Tukey's HSD pairwise comparisons for reproductively inexperienced female chemosensory responses across all concentrations. Significant differences between concentrations are bolded ($P < 0.050$).

Inexp.	0.0 mM	10^{-7} mM	10^{-5} mM	10^{-4} mM	10^{-3} mM	10^{-2} mM (Exp1)	10^{-2} mM (Exp2)	10^{-1} mM	1.0 mM
10^{-7} mM	1.000								
10^{-5} mM	0.345	0.654							
10^{-4} mM	0.857	0.982	0.979						
10^{-3} mM	**0.035**	0.254	1.000	0.711					
10^{-2} mM (Exp1)	0.095	0.474	1.000	0.939	1.000				
10^{-2} mM (Exp2)	0.343	0.671	1.000	0.985	1.000	1.000			
10^{-1} mM	0.062	0.351	1.000	0.840	1.000	1.000	1.000		
1.0 mM	**0.040**	0.221	1.000	0.641	1.000	0.665	0.999	1.000	
2.0 mM	**<0.001**	**<0.001**	0.060	**<0.001**	0.057	**0.007**	**0.037**	**0.028**	0.172

Table 3.1. Ethogram of behaviors used during activity budget observations.

Category	Behavior	Description
Forage	Forage	Acquire, process, and consume food items (e.g. hay, produce, branches, etc.)
Inactive	Stand	Stationary in space; movement less than 1 body length in 3 sec
	Stereotypy	Includes swaying, head bobbing, trunk tossing, and other common stereotypic behaviors
Interaction	Chemosensory	Chemosensory behavior (sniff, check, place, or flehmen) directed towards a chemical sample
	Investigate	Includes sniff air, object, elephant, urine, or feces (not a chemical sample)
	Manipulate	Use trunk, foot, body, or head to interact with non-food items
	Social	Includes affiliative and agonistic behaviors
Self-maintenance	Defecate	Void feces
	Drink	Consume liquid
	Groom	Includes dirt/mud bathe, water bathe, rub, and scratch
	Urinate	Void urine
Walk	Walk	Directed movement from point A to point B; change in location >1 body length in 3 sec
Other	Other behavior	Behavior not listed above; describe in notes
	Out of view	Elephant not visible to observer

Table 3.2. Frontalin observations: results of repeated measures ANOVAs for comparisons of each state behavior between pre-treatment, treatment, and post-treatment phases, categorized by sex and reproductive experienced (Exp. = experienced, Inexp. = inexperienced). Bolded cells indicate statistically significant differences in behavior ($P < 0.050$). "NA" indicates a particular group was never observed engaging in corresponding behavior.

	Males (n = 18)	**Exp. Males** (n = 6)	**Inexp. Males** (n = 12)	**Females** (n = 32)	**Exp. Females** (n = 11)	**Inexp. Females** (n = 21)
Forage	F = 1.006 P = 0.376	F = 0.009 P = 0.991	F = 1.550 P = 0.235	F = 2.125 P = 0.128	F = 1.611 P = 0.225	F = 0.754 P = 0.477
Inactive	**F = 4.912** **P = 0.013**	F = 1.538 P = 0.262	**F = 3.524** **P = 0.047**	F = 0.490 P = 0.615	F = 0.535 P = 0.594	F = 1.172 P = 0.320
Stand	**F = 3.910** **P = 0.030**	F = 1.078 P = 0.377	**F = 4.955** **P = 0.017**	F = 0.381 P = 0.685	F = 0.122 P = 0.886	F = 0.607 P = 0.550
Stereotypy	**F = 3.313** **P = 0.049**	F = 0.763 P = 0.492	F = 3.193 P = 0.061	F = 0.243 P = 0.785	F = 0.456 P = 0.640	F = 0.462 P = 0.633
Interaction	F = 0.164 P = 0.849	F = 0.044 P = 0.957	F = 0.338 P = 0.717	F = 0.206 P = 0.815	F = 0.196 P = 0.824	F = 0.664 P = 0.520
Investigate	F = 0.305 P = 0.739	F < 0.001 P = 1.000	F = 0.371 P = 0.694	F = 0.237 P = 0.790	F = 0.133 P = 0.876	F = 0.682 P = 0.511
Manipulate	F = 1.739 P = 0.191	F = 0.943 P = 0.422	F = 0.897 P = 0.422	F = 1.545 P = 0.221	F = 1.070 P = 0.362	F = 2.776 P = 0.074
Social	F = 0.540 P = 0.588	F = 0.734 P = 0.504	F = 0.140 P = 0.870	F = 0.569 P = 0.569	F = 0.530 P = 0.597	F = 0.557 P = 0.577
Self-maintenance	F = 0.728 P = 0.490	F = 3.532 P = 0.069	F = 1.487 P = 0.248	F = 1.466 P = 0.239	F = 1.508 P = 0.245	F = 0.317 P = 0.730
Defecate	F = 0.486 P = 0.619	F = 0.056 P = 0.946	F = 0.700 P = 0.507	F = 1.072 P = 0.349	F = 1.458 P = 0.256	F = 0.335 P = 0.718
Drink	F = 0.213 P = 0.809	F = 1.000 P = 0.402	F = 1.219 P = 0.315	**F = 5.847** **P = 0.005**	F = 1.454 P = 0.257	**F = 4.389** **P = 0.019**
Groom	F = 1.286 P = 0.289	F = 2.953 P = 0.098	F = 1.248 P = 0.307	F = 2.717 P = 0.074	F = 1.949 P = 0.168	F = 1.037 P = 0.364
Urinate	F = 1.480 P = 0.242	F = 1.383 P = 0.295	F = 0.647 P = 0.533	F = 1.619 P = 0.206	**F = 5.714** **P = 0.011**	F = 0.526 P = 0.595
Walk	**F = 3.903** **P = 0.030**	F = 0.702 P = 0.518	**F = 3.984** **P = 0.033**	F = 1.028 P = 0.364	F = 1.616 P = 0.224	F = 0.514 P = 0.602
Other	F = 0.156 P = 0.857	F = 0.899 P = 0.438	F = 0.053 P = 0.949	**F = 4.726** **P = 0.012**	F = 1.236 P = 0.312	**F = 3.738** **P = 0.033**
Other behavior	F = 0.827 P = 0.446	F = 1.000 P = 0.402	F = 1.000 P =0.384	F = 0.725 P = 0.489	NA	F = 0.721 P = 0.492
Out-of-view	F = 0.882 P = 0.423	F = 1.000 P = 0.402	F = 0.703 P = 0.506	**F = 3.811** **P =0.028**	F = 1.220 P = 0.316	F = 2.604 P = 0.087

Table 3.3. *Z*7-12:Ac observations: results of repeated measures ANOVAs for comparisons of each state behavior between pre-treatment, treatment, and post-treatment phases, categorized by sex and reproductive experienced (Exp. = experienced, Inexp. = inexperienced). Bolded cells indicate statistically significant differences in behavior ($P < 0.050$). "NA" indicates a particular group was never observed engaging in corresponding behavior.

	Males (*n* = 18)	**Exp. Males** (*n* = 6)	**Inexp. Males** (*n* = 12)	**Females** (*n* = 32)	**Exp. Females** (*n* = 11)	**Inexp. Females** (*n* = 21)
Forage	*F* = 1.164 *P* = 0.324	*F* = 2.015 *P* = 0.184	*F* = 3.226 *P* = 0.059	***F* = 3.236** ***P* = 0.046**	*F* = 3.100 *P* = 0.067	*F* = 1.031 *P* = 0.366
Inactive	*F* = 0.236 *P* = 0.791	*F* = 0.840 *P* = 0.460	*F* = 1.535 *P* = 0.238	*F* = 0.016 *P* = 0.984	*F* = 0.513 *P* = 0.606	*F* = 0.588 *P* = 0.560
Stand	*F* = 0.180 *P* = 0.836	*F* = 0.404 *P* = 0.678	*F* = 0.096 *P* = 0.909	*F* = 0.790 *P* = 0.458	*F* = 1.382 *P* = 0.274	*F* = 0.108 *P* = 0.898
Stereotypy	*F* = 0.174 *P* = 0.841	*F* = 0.977 *P* = 0.409	*F* = 1.494 *P* = 0.246	*F* = 1.447 *P* = 0.243	*F* = 0.098 *P* = 0.907	*F* = 2.018 *P* = 0.146
Interaction	*F* = 1.293 *P* = 0.288	*F* = 0.073 *P* = 0.931	*F* = 2.705 *P* = 0.089	*F* = 1.139 *P* = 0.327	*F* = 0.151 *P* = 0.861	*F* = 3.198 *P* = 0.052
Investigate	*F* = 1.103 *P* = 0.344	*F* = 1.480 *P* = 0.273	*F* = 1.271 *P* = 0.300	*F* = 2.774 *P* = 0.070	*F* = 0.036 *P* = 0.964	***F* = 4.771** ***P* = 0.014**
Manipulate	*F* = 1.471 *P* = 0.244	*F* = 0.728 *P* = 0.507	*F* = 1.397 *P* = 0.269	*F* = 1.733 *P* = 0.185	*F* = 0.743 *P* = 0.488	*F* = 1.194 *P* = 0.314
Social	***F* = 3.320** ***P* = 0.048**	*F* = 0.408 *P* = 0.675	***F* = 4.445** ***P* = 0.024**	*F* = 0.522 *P* = 0.596	*F* = 0.395 *P* = 0.679	*F* = 1.233 *P* = 0.302
Self-maintenance	*F* = 2.581 *P* = 0.091	*F* = 1.781 *P* = 0.218	*F* = 1.561 *P* = 0.232	***F* = 4.197** ***P* = 0.020**	*F* = 0.761 *P* = 0.480	***F* = 4.053** ***P* = 0.025**
Defecate	*F* = 0.516 *P* = 0.601	*F* = 2.588 *P* = 0.124	*F* = 0.554 *P* = 0.582	*F* = 0.378 *P* = 0.687	*F* = 0.233 *P* = 0.795	*F* = 0.562 *P* = 0.574
Drink	*F* = 1.668 *P* = 0.204	*F* = 1.000 *P* = 0.402	*F* = 1.253 *P* = 0.305	*F* = 2.783 *P* = 0.070	*F* = 1.567 *P* = 0.233	*F* = 1.480 *P* = 0.240
Groom	*F* = 1.990 *P* = 0.152	*F* = 1.332 *P* = 0.307	*F* = 0.944 *P* = 0.404	***F* = 4.208** ***P* = 0.019**	*F* = 0.671 *P* = 0.522	***F* = 4.223** ***P* = 0.022**
Urinate	*F* = 0.486 *P* = 0.619	*F* = 0.517 *P* = 0.611	*F* = 0.169 *P* = 0.845	*F* = 1.445 *P* = 0.243	*F* = 0.185 *P* = 0.832	*F* = 2.411 *P* = 0.103
Walk	*F* = 2.497 *P* = 0.097	*F* = 0.586 *P* = 0.575	*F* = 2.448 *P* = 0.110	***F* = 3.389** ***P* = 0.040**	*F* = 0.599 *P* = 0.559	***F* = 6.910** ***P* = 0.003**
Other	*F* = 0.613 *P* = 0.548	*F* = 1.000 *P* = 0.402	*F* = 0.081 *P* = 0.923	*F* = 0.427 *P* = 0.654	*F* = 1.405 *P* = 0.269	*F* = 0.136 *P* = 0.873
Other behavior	*F* = 0.383 *P* = 0.685	NA	*F* = 0.376 *P* = 0.691	*F* = 0.957 *P* = 0.390	*F* = 1.000 *P* = 0.386	*F* = 0.909 *P* = 0.411
Out-of-view	*F* = 0.365 *P* = 0.697	*F* = 1.000 *P* = 0.402	*F* = 0.129 *P* = 0.880	*F* = 2.059 *P* = 0.136	*F* = 0.867 *P* = 0.436	*F* = 1.220 *P* = 0.306

Table 3.4. Results of Pearson product moment correlation tests for **(A)** frontalin and **(B)** *Z*7-12:Ac bioassays for reproductively experienced and inexperienced males and females in different behavioral states. Bolded cells indicate statistically significant findings ($P < 0.050$).

(A) Frontalin

	Inactive	**Interaction**	**Walk**
Male ($n = 18$)	$r = 0.179$ $P = 0.477$	$r = 0.218$ $P = 0.386$	$r = 0.288$ $P = 0.247$
Experienced ($n = 6$)	$r = -0.281$ $P = 0.589$	$r = 0.022$ $P = 0.966$	$r = 0.043$ $P = 0.935$
Inexperienced ($n = 12$)	$r = 0.483$ $P = 0.112$	$r = 0.298$ $P = 0.348$	$r = 0.422$ $P = 0.172$
Female ($n = 32$)	$r = -0.242$ $P = 0.181$	$r = 0.237$ $P = 0.192$	$r = 0.163$ $P = 0.373$
Experienced ($n = 11$)	$r = -0.403$ $P = 0.219$	$r = 0.532$ $P = 0.092$	**$r = 0.726$** **$P = 0.011$**
Inexperienced ($n = 21$)	$r = -0.158$ $P = 0.495$	$r = 0.136$ $P = 0.558$	$r = 0.130$ $P = 0.573$

(B) *Z*7-12:Ac

	Inactive	**Interaction**	**Walk**
Male ($n = 18$)	$r = -0.182$ $P = 0.470$	$r = -0.201$ $P = 0.423$	**$r = 0.481$** **$P = 0.044$**
Experienced ($n = 6$)	$r = -0.098$ $P = 0.854$	$r = -0.377$ $P = 0.461$	**$r = 0.824$** **$P = 0.044$**
Inexperienced ($n = 12$)	$r = -0.230$ $P = 0.471$	$r = -0.163$ $P = 0.613$	$r = 0.219$ $P = 0.495$
Female ($n = 32$)	**$r = -0.357$** **$P = 0.045$**	**$r = 0.480$** **$P = 0.005$**	$r = 0.117$ $P = 0.522$
Experienced ($n = 11$)	$r = -0.073$ $P = 0.832$	$r = 0.530$ $P = 0.094$	$r = -0.122$ $P = 0.720$
Inexperienced ($n = 21$)	**$r = -0.460$** **$P = 0.036$**	**$r = 0.477$** **$P = 0.029$**	$r = 0.140$ $P = 0.546$

Table 3.5. The effects of frontalin and *Z*7-12:Ac on elephant behavior, as anecdotally reported by keepers. 'Exp.' column indicates Experiment 1 (mid-range concentrations) or 2 (low and high concentrations). 'Facility' and 'Subject' columns correspond to information presented in Appendix I. 'Repro. Exper.' indicates reproductively experienced ('Exp.') or inexperienced ('Inexp.') elephants.

Exp.	Compound	Facility	Subject	Sex	Age (yr)	Repro. Exper.	Notes
1	Frontalin	4	13	F	46	Exp.	Unusual vocalizations; dug holes where samples were placed
1	Frontalin	4	15	F	32	Exp.	Dug holes where samples were placed
1	Frontalin	6	28	M	24	Inexp.	Different walking patterns around yard
1	*Z*7-12:Ac	7	34	M	48	Exp.	Musth-like symptoms; uncooperativeness
1	*Z*7-12:Ac	8	41	M	45	Exp.	Abnormally long musth; increased semen quality during collections
2	*Z*7-12:Ac	11	53	F	48	Inexp.	Aversion; more than disinterest
2	*Z*7-12:Ac	12	57	M	31	Exp.	Unusual investigatory and locomotor behavior
2	Frontalin	14	65	M	7	Inexp.	Unusual interest in own urine
2	Frontalin	5	67	M	11	Inexp.	Unusual interest in own urine (including flehmens)
2	Frontalin	5	68	M	17	Inexp.	Unusual interest in own urine
2	Frontalin	5	26	F	11	Inexp.	Unusual vocalizations and heightened wariness
2	Frontalin	5	76	M	43	Exp.	Unusual interest in own urine (including flehmens)

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APPENDIX I

SAMPLE POPULATION PARAMETERS

Table A1.1. Details on each elephant included in Experiment 1 bioassays (mid-range concentrations, from 10^{-4} to 10^{-1} mM of each compound) and activity budget observations. Groups were denoted in the order that elephants were observed on the first day of bioassays. Animals in the same group were observed simultaneously in the same space. 'Studbook' column indicates studbook numbers for each animal assigned by the Association of Zoos and Aquariums' Asian Elephant Species Survival Plan® (Keele, 2015). Sex is male ('M') or female ('F'). Age column is noted in years, on first day of observations. Reproductive status (if known from hormonal records less than 1 yr old) is noted; if female is cyclic, hormonal data confirm luteal or follicular phase. For females, parity is 'natural' (occurring through natural breeding), 'AI' (occurring through artificial insemination), 'N/A' (indicating the female is pre-cyclic, or considered too young to breed), or nulliparous. Start date of a five-day experiment is indicated in the last column. In 'Subject' column, * indicates elephants also observed in experiment 2, and § indicates elephants that were excluded from activity budget observations.

Facility	Group	Subject	Studbook	Sex	Age (yr)	Reproductive status	Parity	Start date
1	1A	1	268	F	33.6	Cyclic (unknown phase)	Nulliparous	11 May 2015
	1B	2	269	F	32.4	Cyclic (unknown phase)	Nulliparous	11 May 2015
2	2A	3	535	M	14.1	Pre-musth	--	18 May 2015
	2B	4	518	M	15.6	Pre-musth	--	18 May 2015
	2B	5	687	M	5.5	Pre-musth	--	18 May 2015
	2B	6	653	M	6.8	Pre-musth	--	18 May 2015
3	3A	7	420	M	18.1	Non-musth	--	25 May 2015
	3B	8	368	F	19.9	Pregnant	Natural	25 May 2015
	3B	9	26	F	39.5	Cyclic (luteal phase)	Natural	25 May 2015
4	4A	10*	126	M	50.4	Non-musth	--	03 Jun 2015
	4A	11	632	M	10.1	Non-musth	--	03 Jun 2015
	4A	12	671	M	5.1	Pre-musth	--	03 Jun 2015
	4B	13	127	F	46.4	Cyclic (luteal phase)	Natural	03 Jun 2015
	4B	14	308	F	24.6	Cyclic (luteal phase)	Natural	03 Jun 2015
	4B	15	288	F	32.4	Cyclic (follicular phase)	Natural	03 Jun 2015

Table A1.1, continued.

Facility	Group	Subject	Studbook	Sex	Age (yr)	Reproductive status	Parity	Start date
4 (cont.)	4B	16	735	F	4.7	Cyclic (follicular phase)	N/A	03 Jun 2015
	4B	17	760	M	1.3	Pre-musth	--	03 Jun 2015
5	5A	18	539	F	13.4	Cyclic (follicular phase)	Nulliparous	11 Jun 2015
	5A	19*	655	F	6.6	Cyclic (unknown phase)	Nulliparous	11 Jun 2015
	5B	20*	739	F	2.8	Cyclic (unknown phase)	N/A	11 Jun 2015
	5C	21	199	F	39.4	Cyclic (luteal phase)	Natural	11 Jun 2015
	5D	22*	546	M	13.1	Non-musth	--	16 Jun 2015
	5E	23*	537	M	13.6	Non-musth	--	16 Jun 2015
	5F	24*	656	M	6.4	Pre-musth	--	16 Jun 2015
	5G	25	117	F	54.5	Unknown	Nulliparous	22 Jun 2015
	5H	26*	633	F	10.2	Cyclic (unknown phase)	Nulliparous	22 Jun 2015
	5I	27*	115	F	39.5	Unknown	Nulliparous	22 Jun 2015
6	6A	28	327	M	24.3	Non-musth	--	13 Jul 2015
	6B	29	179	F	44.5	Cyclic (follicular phase)	Natural	13 Jul 2015
	6B	30	302	F	26.5	Cyclic (follicular phase)	Nulliparous	13 Jul 2015
	6B	31	515	F	16.6	Cyclic (luteal phase)	AI	13 Jul 2015
	6B	32	750	F	2.0	Pre-cyclic	N/A	13 Jul 2015
	6B	33	758	M	1.9	Pre-musth	--	13 Jul 2015
7	7A	34	263	M	47.6	Non-musth	--	20 Jul 2015
	7B	35	365	F	20.5	Acyclic (lactating)	Natural	20 Jul 2015
	7B	36	385	F	19.1	Cyclic (unknown phase)	Nulliparous	20 Jul 2015
	7B	37	246	F	36.6	Cyclic (unknown phase)	AI	20 Jul 2015
	7B	38	245	F	48.7	Cyclic (unknown phase)	Nulliparous	20 Jul 2015
	7B	39§	736	F	4.3	Unknown	N/A	20 Jul 2015
	7B	40§	764	F	0.6	Pre-cyclic	N/A	20 Jul 2015
8	8A	41	160	M	44.6	Musth	--	27 Jul 2015
	8B	42	239	F	64.7	Acyclic	Nulliparous	27 Jul 2015
	8B	43	159	F	43.1	Acyclic	Nulliparous	27 Jul 2015

Table A1.1, continued.

Facility	Group	Subject	Studbook	Sex	Age (yr)	Reproductive status	Parity	Start date
9	9A	44	76	F	41.2	Cyclic (unknown phase)	Nulliparous	03 Aug 2015
	9B	45	27	M	43.6	Non-musth	--	03 Aug 2015
	9B	46	42	F	34.0	Cyclic (unknown phase)	Natural	03 Aug 2015
10	10A	47	339	M	22.6	Non-musth	--	10 Aug 2015
	10B	48	247	F	35.6	Cyclic (luteal phase)	Natural	10 Aug 2015
	10B	49	234	F	44.6	Acyclic	Natural	10 Aug 2015
	10B	50	235	F	44.4	Acyclic	Nulliparous	10 Aug 2015
	10B	51	642	F	9.0	Cyclic (luteal phase)	Nulliparous	10 Aug 2015
	10B	52	646	F	8.5	Pre-cyclic	N/A	10 Aug 2015

Table A1.2. Details on each elephant included in Experiment 2 bioassays (low and high concentrations, from 10^{-7} to 2.0 mM of each compound). Groups were assigned in the order that elephants were denoted on the first day of bioassays. Animals in the same group were observed simultaneously in the same space. 'Studbook' column indicates studbook numbers for each animal assigned by the Association of Zoos and Aquariums' Asian Elephant Species Survival Plan® (Keele, 2015). Sex is male ('M') or female ('F'). Age column is noted in years, on first day of observations. Reproductive status (if known from hormonal records less than 1 yr old) is noted; if female is cyclic, hormonal data confirm luteal or follicular phase. For females, parity is 'natural' (occurring through natural breeding), 'AI' (occurring through artificial insemination), 'N/A' (indicating the female is pre-cyclic, or considered too young to breed), or nulliparous. The date the bioassays occurred (or the first day of bioassays if conducted over multiple days) is indicated in the last column. ***** in 'Subject' column indicates elephants observed in experiment 1 as well.

Facility	Group	Subject	Studbook	Sex	Age (yr)	Reproductive status	Parity	Start date
4	4C	10*	126	M	51.0	Non-musth	--	15 Dec 2015
11	11A	53	132	F	48.0	Cyclic (unknown phase)	Nulliparous	13 Jan 2016
	11A	54	282	F	32.0	Cyclic (unknown phase)	Natural	13 Jan 2016
12	12A	55	673	F	52.0	Cyclic (unknown phase)	Nulliparous	15 Jan 2016
	12A	56	672	F	50.0	Cyclic (unknown phase)	Nulliparous	15 Jan 2016
	12B	57	309	M	31.0	Musth	--	16 Jan 2016
13	13A	58	221	F	39.1	Cyclic (unknown phase)	Nulliparous	19 Jan 2016
	13A	59	214	F	52.1	Acyclic	Natural	19 Jan 2016
	13A	60	220	F	49.1	Acyclic	Nulliparous	19 Jan 2016
	13B	61	218	M	49.4	Non-musth	--	19 Jan 2016
14	14A	62	216	F	43.1	Acyclic	Nulliparous	30 Jan 2016
	14A	63	353	F	28.7	Cyclic (unknown phase)	Natural	30 Jan 2016
	14A	64	276	M	28.0	Non-musth	--	30 Jan 2016
	14B	65	657	M	6.9	Pre-musth	--	31 Jan 2016
5	5E	23*	537	M	14.3	Non-musth	--	22 Mar 2016
	5F	24*	656	M	7.2	Pre-musth	--	22 Mar 2016
	5J	66	260	M	32.1	Non-musth	Castrated	22 Mar 2016
	5K	67	634	M	10.8	Non-musth	--	22 Mar 2016
	5L	68	526	M	16.6	Musth	--	22 Mar 2016

Table A1.2, continued.

Facility	Group	Subject	Studbook	Sex	Age (yr)	Reproductive status	Parity	Start date
5 (cont.)	5M	69	503	M	45.2	Non-musth	--	22 Mar 2016
	5D	22*	546	M	13.8	Non-musth	--	24 Mar 2016
	5N	70	249	F	42.2	Pregnant	Natural	24 Mar 2016
	5O	27*	115	F	40.2	Unknown	Nulliparous	24 Mar 2016
	5O	71	107	F	38.2	Unknown	Nulliparous	24 Mar 2016
	5H	26*	633	F	10.9	Cyclic (unknown phase)	Nulliparous	24 Mar 2016
	5P	72	240	M	53.2	Musth	--	24 Mar 2016
	5Q	73	502	M	43.2	Non-musth	--	24 Mar 2016
	5R	19*	655	F	7.4	Cyclic (unknown phase)	Nulliparous	26 Mar 2016
	5R	20*	739	F	3.6	Cyclic (unknown phase)	--	26 Mar 2016

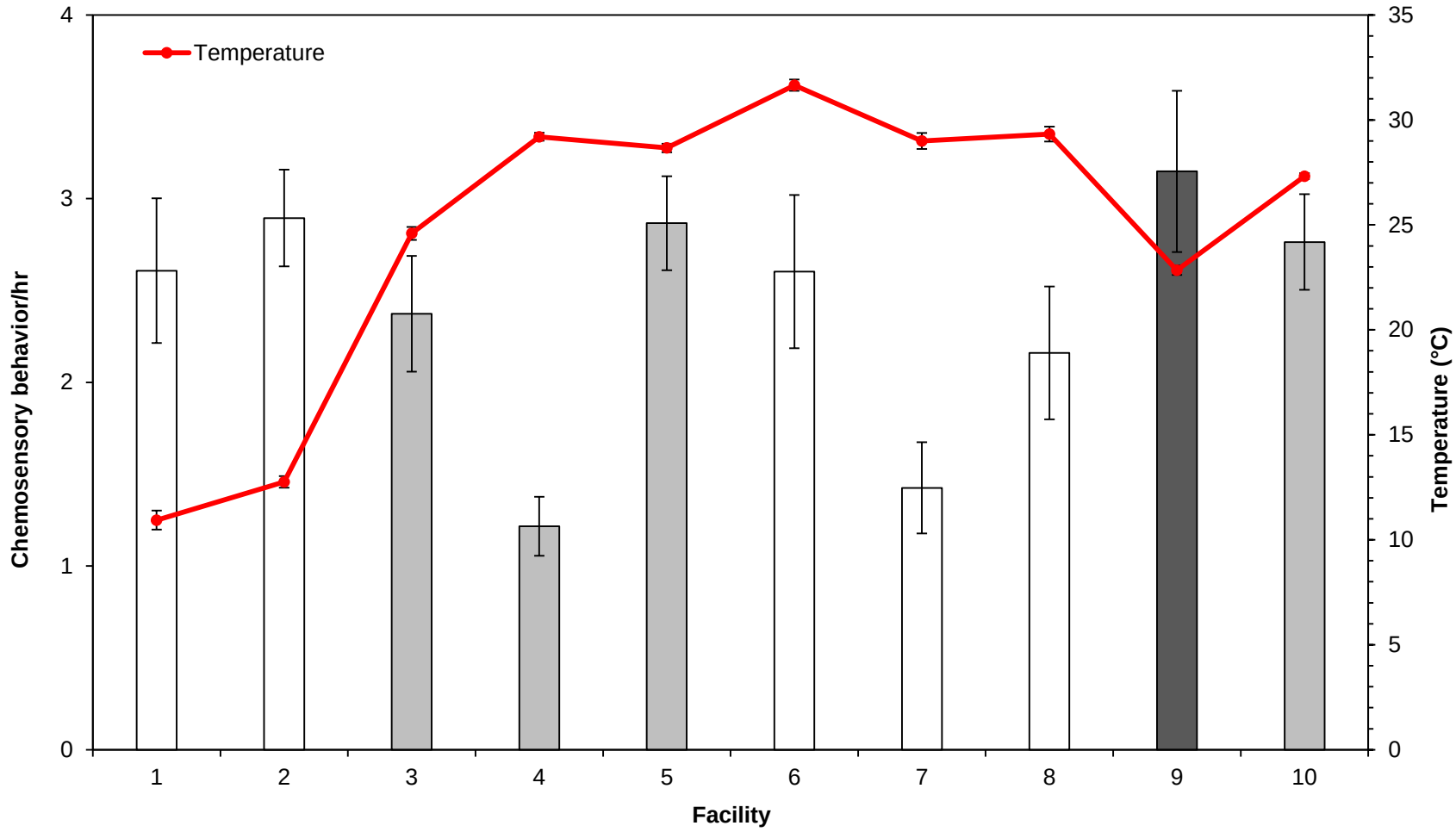

Figure A1.1. Mean ± SE chemosensory response to samples by facility by substrate: packed dirt (white bars, □), loose sand (light gray, ▒), and unpolished concrete (dark gray, ■). Average temperature ± SE by each facility is shown by a red line. Facilities are ordered on the horizontal axis by date of visit (for first experiment bioassays only).

APPENDIX II

CHEMICAL PROPERTIES OF *Z*7-12:Ac AND FRONTALIN

Introduction

Chemical ecologists utilize a variety of technology to analyze chemical signals. One technique, gas chromatography–mass spectrometry (GC-MS), allows for the detection and identification of a variety of chemical signals (e.g. Nojima, Schal, Webster, Santangelo, & Roelofs, 2005; Rasmussen, Lee, et al., 1997; Schaal et al., 2003). A gas chromatograph utilizes a column to separate a sample into its respective components. These components are captured downstream by a mass spectrometer for identification by retention time. In this way, it is possible to identify the component parts of secretions directly collected from organisms, and then, each component can be bioassayed to test for its relative bioactivity. GC-MS can also be used to validate protocols when synthetic sources of signals are used.

The objectives of the following experiments were to analyze via GC-MS the capability of *Z*7-12:Ac and frontalin to remain in sodium phosphate buffer over an extended period of time, and also to use GC-MS to analyze quantitative differences in *Z*7-12:Ac and frontalin samples of different concentrations.

Methods

Chemical samples were prepared and stored before analysis as described in Chapter 2 of this work; samples were prepared less than 1 hour before GC-MS analysis.

Two separate experiments were conducted for these analyses. The first analyzed the amount of either compound in a sample every hour for 21 hours (for bioassays in this

work, no samples were prepared more than 18 hours before they were poured). For the first experiment, I analyzed 10^{-1} mM of *Z*7-12:Ac and frontalin to ensure detection by the mass spectrometer. The second experiment compared the 10^{-1} mM and 10^{-2} mM samples of each compound every hour for five hours to ensure that the sodium phosphate buffer was not degrading the compounds, and that there was a distinct chemical difference in concentration between the samples. Again, these concentrations were chosen to ensure detection by the mass spectrometer. In both experiments 5 mL of both compounds were present simultaneously in the same 20 mL glass vial for analysis. For the second experiment, two separate vials were used: one contained 5 mL of each compound at 10^{-1} mM, and the other contained each compound at 10^{-2} mM.

Analyses of chemical samples were conducted via headspace gas chromatography on a Varian 450-GC gas chromatograph and a Varian 220-MS IT mass spectrometer (Hachenberg & Schmidt, 1977). A custom protocol involved headspace injection of 500μL from each vial via an autosampler with a gas-tight syringe. Electron impact mass spectra were recorded in the 10–650 amu range to identify *Z*7-12:Ac (MW = 226.36) and frontalin (MW = 142.2). Separation was performed on a Phenomenex, Inc. Zebron™ column with a ZB-5MS stationary phase (30 m with 0.25 mm diameter). The temperature program was isothermal at 35°C for 5 min, then raised to 280°C at 5°C min^{-1} and held for 0 min. This program was repeated each hour as many times as necessary (22 hours for the first experiment and 5 hours for the second experiment).

Identification of both *Z*7-12:Ac and frontalin was achieved through mass spectrometry of known peaks stored in the National Institute of Standards and

Technology (NIST) Library database. The amount of each compound in the samples were determined by calculating the area of each peak.

Results

The amounts of each 5 mL sample of 10^{-1} mM *Z*7-12:Ac (Figure A2.1) and 10^{-1} mM frontalin (Figure A2.2) did not vary substantially over 22 hours. The amount of *Z*7-12:Ac over 22 hours in the samples ranged from 69,600 – 120,100; frontalin ranged from 6,218 – 8,711. Neither compound showed clear increasing or decreasing patterns over time. Each compound was analyzed approximately 120 hours later, and both *Z*7-12:Ac (81,866) and frontalin (6,507) were present in similar amounts to those tested over the 22-hr experiment.

In the second experiment, there was no notable difference in the amount of Z7-12:Ac between the 10^{-1} mM and 10^{-2} mM samples: the average ± SD amount of *Z*7-12:Ac present in the 10^{-1} mM sample over 5 hours was 76,712 ± 9,068; the average ± SD amount in the 10^{-2} mM sample was 71,973 ± 5,374 (Figure A2.3). However, there was a marked difference in the two concentrations of frontalin tested. The average ± SD amount of frontalin in the 10^{-1} mM sample was 8,377 ± 1,005, while the average ± SD amount in the 10^{-2} mM sample was only 934 ± 118 (Figure A2.4).

Discussion

The first experiment confirmed that neither *Z*7-12:Ac nor frontalin degraded in buffer over 21 hours; apparently it is also feasible to keep either in buffer for at least five days, as I obtained similar amounts after 120 hours to the 21-hr experiment. Certainly, it

does not seem that either compound degraded in buffer in the time between making the samples and performing any of the bioassays during this project (a span that was at most 18 hours).

However, the second experiment yielded results that led to more questions. The difference between the 10^{-1} mM and 10^{-2} mM frontalin samples is clear: over 5 hrs, the 10^{-2} mM sample contained approximately 10% of the compound as the 10^{-1} mM did. This is also evident in the mass spectra (Figure A2.5). The mass spectra of the *Z*7-12:Ac do not show this pattern (Figure A2.6). Instead, headspace analysis yielded similar amounts across all time points for the 10^{-1} mM and 10^{-2} mM *Z*7-12:Ac samples.

It is unclear why the *Z*7-12:Ac samples did not show clear differences in concentrations similar to the frontalin samples. These results may indeed be anomalous, as samples were only tested once. Headspace gas chromatography was chosen because it allows for minute amounts of volatile compounds to be measured, especially for those in a water-based matrix (Goodwin & Schulte, 2009; Kolb & Ettre, 2006). Instead of sampling directly from the liquid, this technique analyzes the gas ('headspace') that volatizes directly above the liquid. There is also greater relevance in using headspace gas chromatography in this project, as many behaviors (e.g. sniffs) took place above each chemical samples, not in the samples themselves. However, it is possible that when the compounds volatilized, they went back into solution due to the high concentration of *Z*7-12:Ac in the headspace above. I chose to analyze relatively high concentrations of each compound (10^{-1} and 10^{-2} mM) to ensure that frontalin could be detected readily and consistently. These concentrations may have been high enough to reach saturation in the headspace for each of the *Z*7-12:Ac samples, yielding the observed results. With more

precise instrumentation, it may be possible to test this idea with lower concentrations of *Z*7-12:Ac. However, the boiling point of frontalin (approximately 60°C) is considerably lower than that of *Z*7-12:Ac (approximately 105°C), indicating that frontalin should have been more volatile in the vial. Boiling points are crude indicators of volatility—especially with largely unknown substances—but frontalin is still considered to be more volatile than *Z*7-12:Ac. Insufficient resources did not allow for validation trials, but further investigation is certainly warranted.

Still, these results affirm the chemical preparation protocols used in this project: both compounds persisted in buffer for an extended period, and (at least for frontalin) there were distinguishable chemical signatures between concentrations. Further experiments should investigate similar properties of both *Z*7-12:Ac and frontalin on various substrates (e.g. dirt, sand, concrete) and in different conditions (e.g. varying temperature, humidity) to ensure that concentration effects are still detectable via GC-MS, thereby validating bioassay protocols for similar projects.

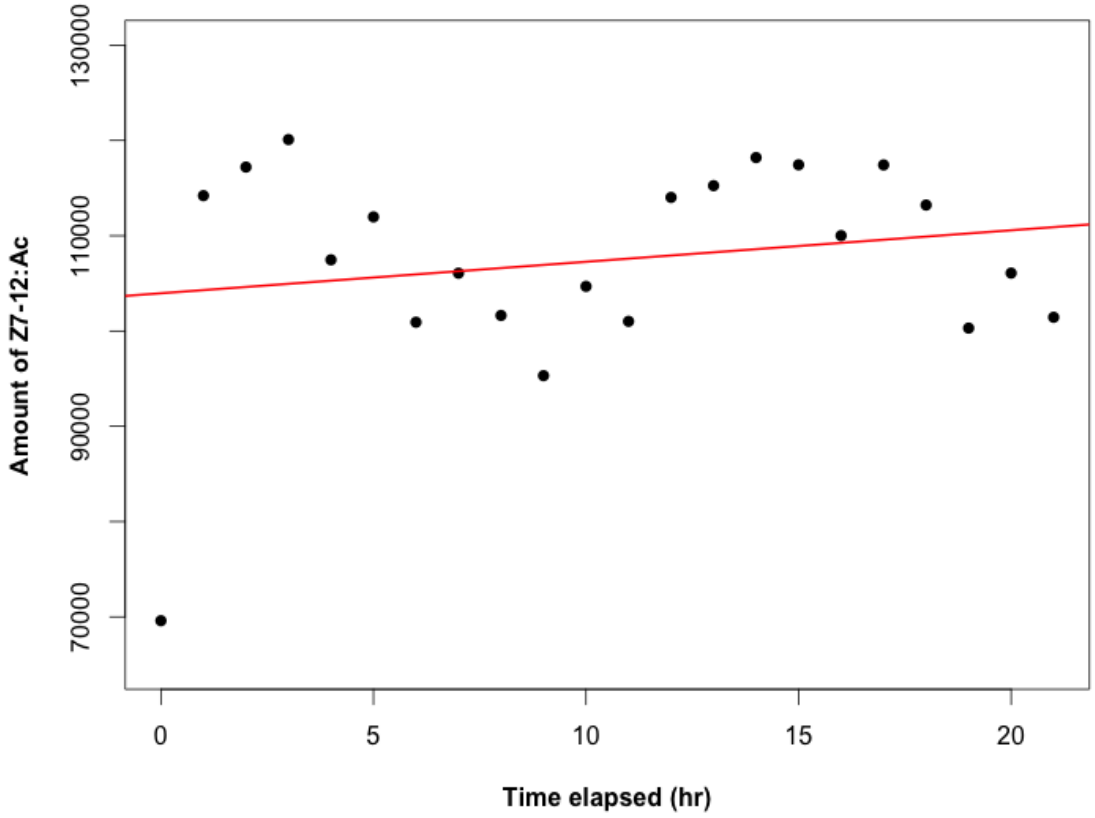

Figure A2.1. Amount of *Z*7-12:Ac present in 5 mL of 10^{-1} mM sample over 21 hours, as measured by GC-MS headspace analysis. Red line shows linear regression.

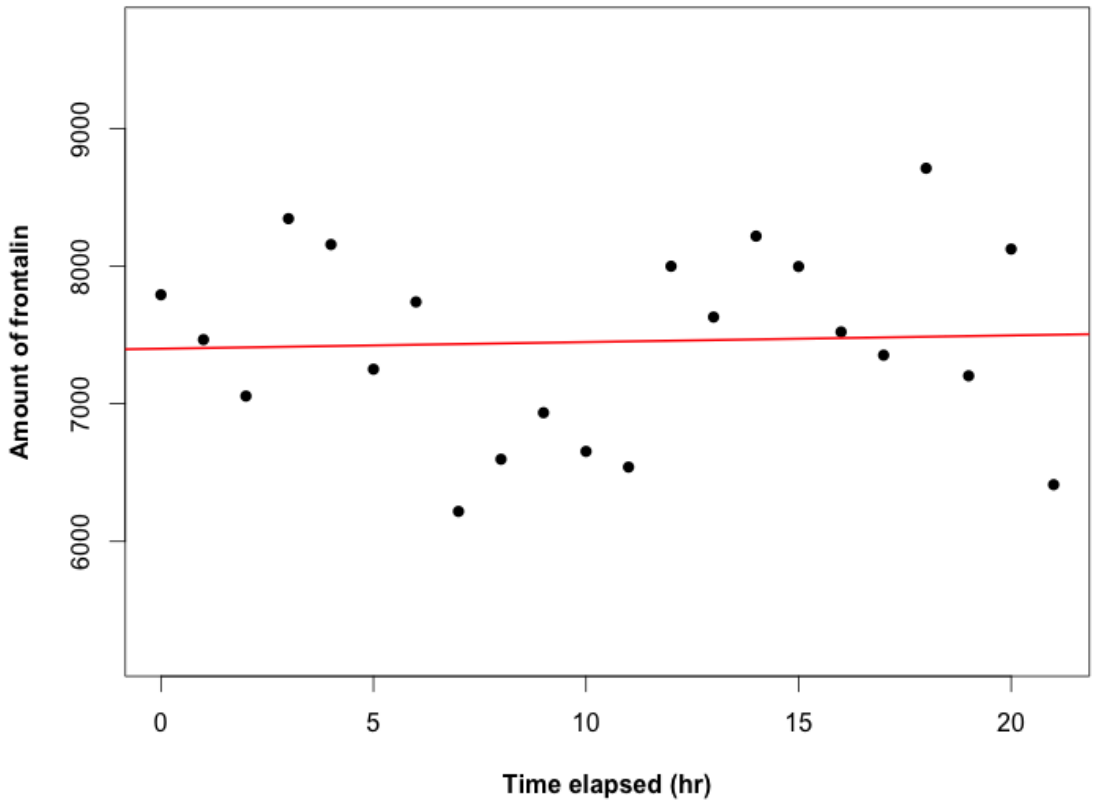

Figure A2.2. Amount of frontalin present in 5 mL of 10^{-1} mM sample over 21 hours, as measured by GC-MS headspace analysis. Red line shows linear regression.

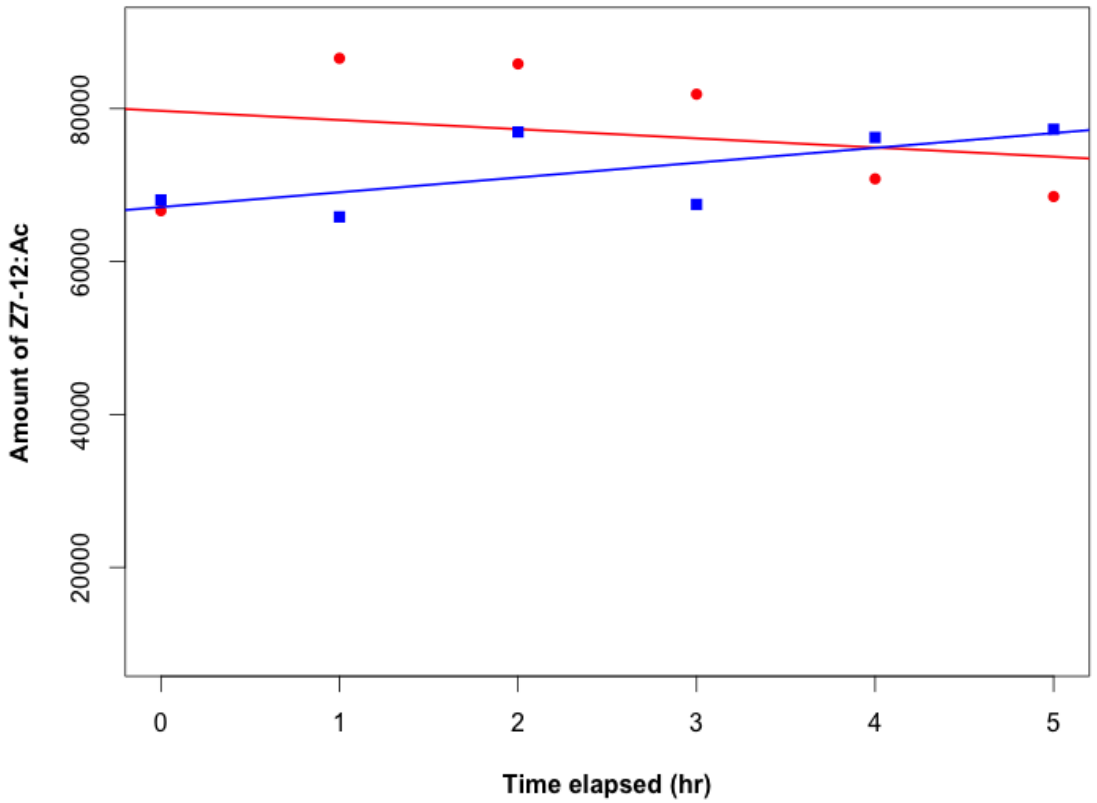

Figure A2.3. Amount of *Z*7-12:Ac present in 5 mL each of 10^{-1} mM sample (blue squares) and 10^{-2} mM sample (red circles) over 5 hours, as measured by GC-MS headspace analysis. Blue line shows linear regression for the 10^{-1} mM sample; red line shows linear regression for the 10^{-2} mM sample.

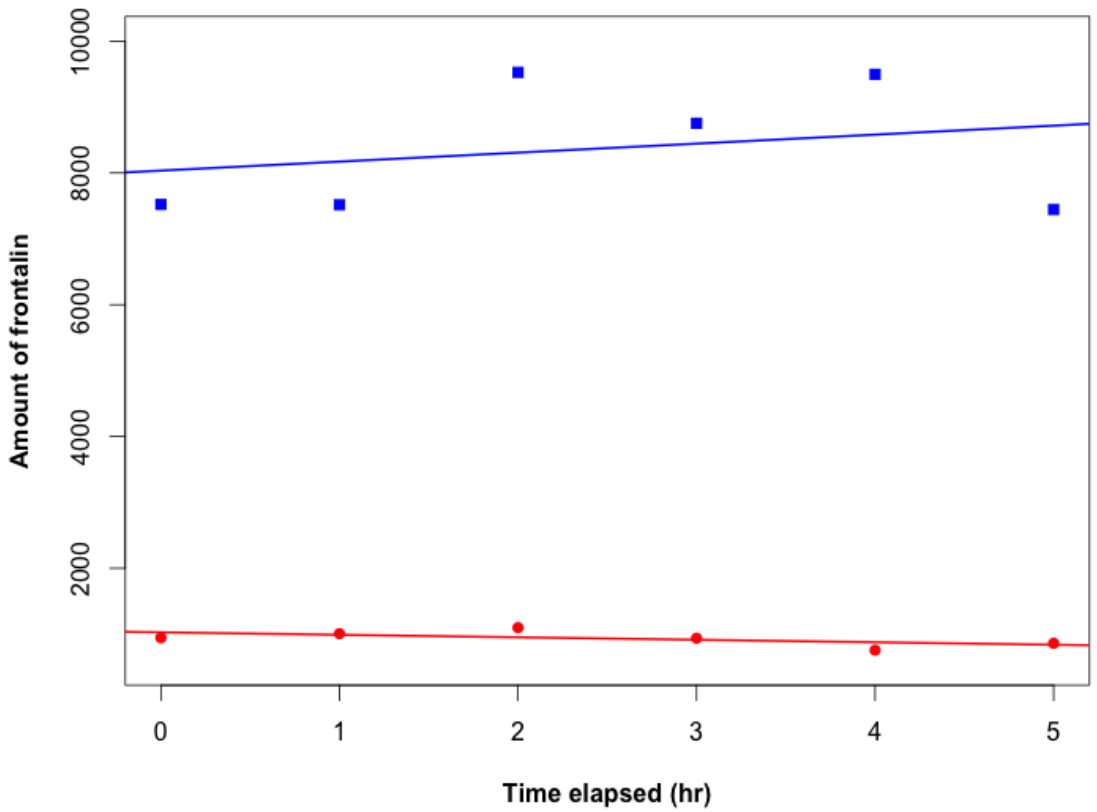

Figure A2.4. Amount of frontalin present in 5 mL each of 10^{-1} mM sample (blue squares) and 10^{-2} mM sample (red circles) over 5 hours, as measured by GC-MS headspace analysis. Blue line shows linear regression for the 10^{-1} mM sample; red line shows linear regression for the 10^{-2} mM sample.

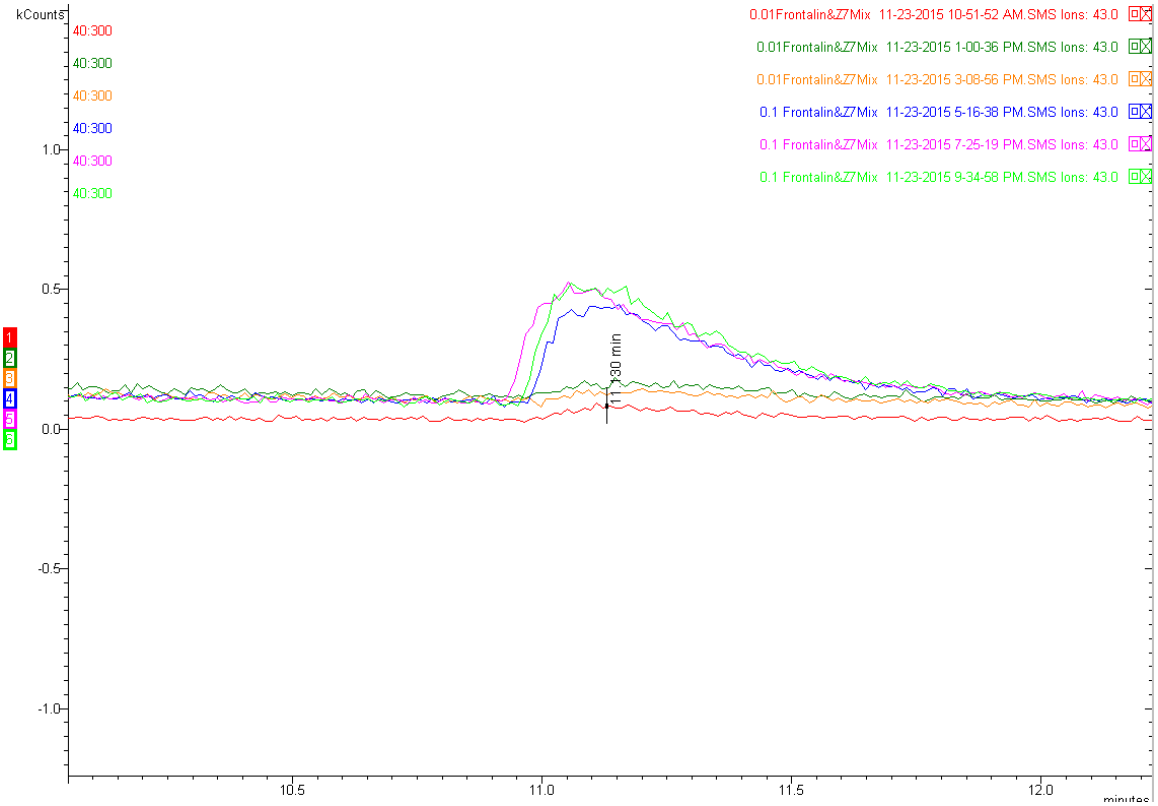

Figure A2.5. Mass spectra of the frontalin samples, analyzed approximately every two hours. The red (■, t = 0 hr), dark green (■, t = 2 hr), and orange (■, t = 4 hr) lines indicate the three 10^{-2} mM samples in succession. The blue (■, t = 0 hr), pink (■, t = 2 hr), and light green (■, t = 4 hr) lines indicate the three 10^{-1} mM samples.

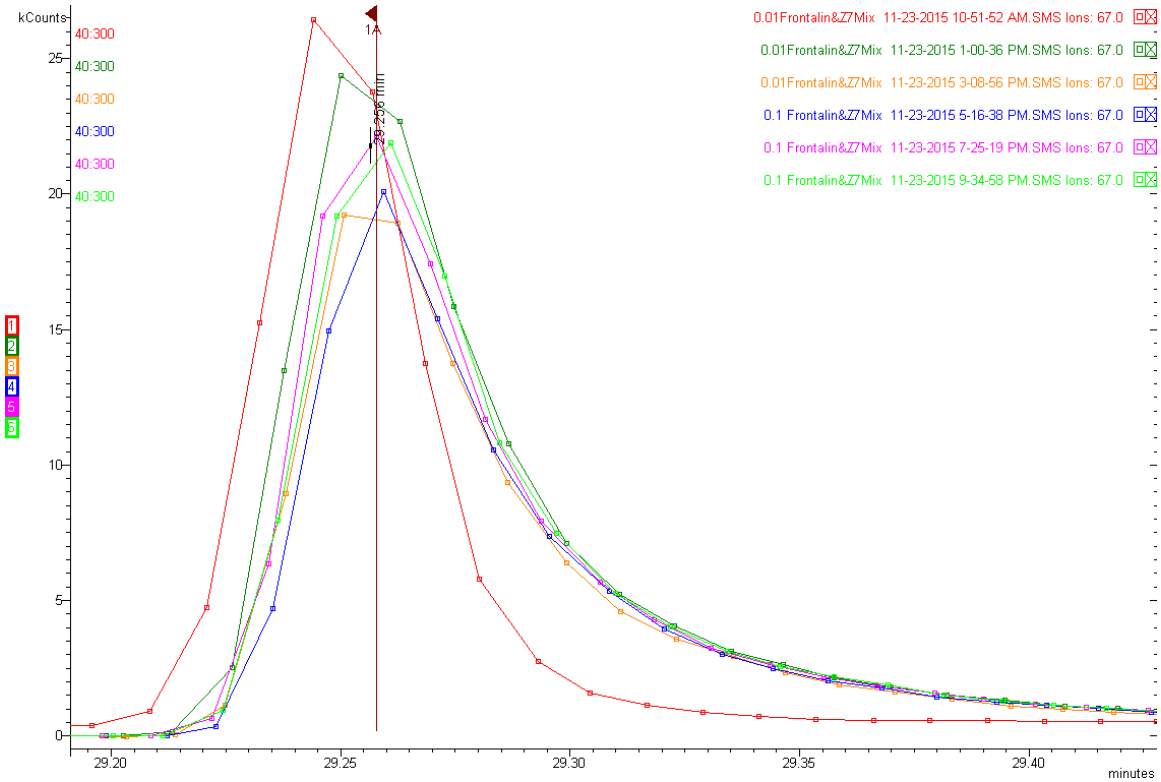

Figure A2.6. Mass spectra of the *Z*7-12:Ac samples, analyzed approximately every two hours. The red (■, t = 0 hr), dark green (■, t = 2 hr), and orange (■, t = 4 hr) lines indicate the three 10^{-2} mM samples in succession. The blue (■, t = 0 hr), pink (■, t = 2 hr), and light green (■, t = 4 hr) lines indicate the three 10^{-1} mM samples.