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Filter efficiency testing for application to bioisolation systems on space station Freedom: Bioassay of chemical signal (pheromone/odor) transmission

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on Space Station Freedom: Bioassay of chemical signal
(pheromone/odor) transmission**

Norris, Nathan, M.A.

San Jose State University, 1993

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FILTER EFFICIENCY TESTING
FOR APPLICATION TO BIOISOLATION SYSTEMS ON
SPACE STATION FREEDOM: BIOASSAY OF CHEMICAL SIGNAL
(PHEROMONE / ODOR) TRANSMISSION

A Thesis

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The Faculty of the Department of Biological Sciences

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In Partial Fulfillment

of the Requirements for the Degree

Master of Arts

by

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ABSTRACT

FILTER EFFICIENCY TESTING FOR APPLICATION TO BIOISOLATION SYSTEMS ON SPACE STATION FREEDOM: BIOASSAY OF CHEMICAL SIGNAL (PHEROMONE / ODOR) TRANSMISSION

by Nathan Norris

Many mammalian pheromonal effects, such as modulation of the timing of the rat's estrus cycle by airborne chemical signals (pheromones) have been demonstrated. This is of particular interest to designers of animal habitats intended for use in space (microgravity) in terms of whether such airborne signals can be blocked by filtration. In this study a pheromonal bioassay associated with estrus cycle entrainment was utilized, along with other measures, to determine the filtering efficiency of a prototype filter. Although not statistically significant (Chi Square, $p < .75$), a trend was noted in the estrus status of two groups exposed to air theoretically containing pheromones. Animals exposed to filtered air did not exhibit the same degree of cycle entrainment as unfiltered air recipients (19% vs. 29%) which may be indicative of effective filtering. Additionally, the filters removed odors, ammonia, waste gases, and contained urine, feces and rat dander from six rats for thirty-one days.

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INTRODUCTION

The timing of estrus in the rat can be modulated by airborne chemical signals or pheromones from other female rats (McClintock, 1978, 1984; McClintock and Adler, 1978). Such effects can confound experimental results if not controlled, especially between animals in different experiments located in close proximity. This is of particular importance in the design of rodent habitats in sealed environments utilizing recirculated air and/or side by side rodent housing modules in which separate experiments may take place. Bioisolation of the crew compartment from the animal habitats is also a serious concern. Currently used animal habitats, such as the animal enclosure module (AEM) used on the space shuttle, are not well bioisolated from the crew compartment. The deficiencies in the bioisolation system has allowed contaminants from the AEM's to escape into the crew compartment resulting in complaints of rat urine and feces odors (Beck, 1992).

In order to eliminate these and other confounding factors in the animal habitats intended for the Centrifuge Facility of Space Station Freedom, NASA requires that the animal modules be bioisolated from each other and that the habitat as a whole be bioisolated from the crew compartment (NASA, 1992). Accordingly, both the inlet and outlet air of the rodent habitat modules will be filtered. A custom made filter incorporating a High Efficiency Particulate Air (HEPA) layer (exclusion limit: 0.3 μ m), among others, was designed by McDonnell Douglas Space Systems Company and the Pall Corporation for this application. The filter was designed to meet requirements including the capability to block the transmission of most bioactive contaminants and withstand the waste load (urine and feces) and dander load of animals in microgravity for a minimum of 14 days

(NASA, 1992).

The purpose of this project was to evaluate aspects of this prototype filter specifically for: (1) ability to block the transmission of odors including chemical signals (pheromones) throughout a 14 day period, (2) ability to block the transmission of ammonia and other gaseous contaminants throughout a 14 day period, and (3) ability to withstand animal waste load and dander load for a minimum of 14 days. The hypothesis that was tested was that these specially designed filters were capable of blocking transmission of all of the above mentioned bioactive contaminants while remaining functional under the animal waste load. This could be demonstrated by the absence of key constituents of animal waste gases including pheromones in the filtered air.

In order to test this hypothesis, several component studies were conducted. The primary goal of this report is to address the effectiveness of the filters in blocking chemical signal (pheromonal) transmission; however, all components of the project performed at San Jose State University will be reported. The filters were designed to block the transmission of most airborne bioactive contaminants including pheromones, odors, ammonia, microbes, and other waste gases. The ability of these filters to effectively block pheromone transmission was assessed by the presence or absence of pheromones in the filtered air. The presence of pheromones may be determined using a known observable change in the timing of the rat's estrus cycle as a marker. The first component of this project (titled: Bioassay Standardization) was a bioassay standardization study to evaluate the protocol and to confirm that an effect of chemical signal (pheromone) transmission could be demonstrated as previously reported in the literature (McClintock, 1978, 1984; McClintock and Adler, 1978). The second component of the study (titled: Pheromone

Filtration Bioassay / Filter Performance) was the next logical step, involving the placement of a filter in the airflow carrying the chemical signals (pheromones) to see if the effect elicited by the pheromones could be blocked. This experiment utilized a McDonnell Douglas test fixture featuring a rodent habitat with a specimen chamber that had the same dimensions as the Centrifuge Facility specimen habitat intended for Space Station Freedom (Strength et al., 1993). During this phase of the project, ammonia was monitored, air samples (for gas chromatography and odor assessment) were collected to assess transmission of airborne materials, and pressure drops across the filters were recorded to assess the filters' ability to withstand waste loading. These measures were also made during a second simplified replication of this component of the project. A human panel performed an odor assessment (sniff) test to detect odor transmission across the filter.

LITERATURE REVIEW - PHEROMONES

I. The Discovery of Pheromones

Chemical signalling between animals has been recognized for thousands of years and their existence is suggested in writings dating back to the Roman era (Agosta, 1992; Norris, 1980). The marking behavior of male dogs, the attraction of male dogs to bitches in heat, and even the importance of masking the odor of the hunter during a hunt are simple examples of the significance of chemical signalling. Never-the-less, it wasn't until the 19th century that serious scientific investigation of insect pheromones began. Understanding came slowly and in the 1930's convincing evidence indicated that volatile

chemical substances were responsible for the attraction of female silkworm moths to the male (Agosta, 1992). In 1956, after over 20 years of work, the German chemist and Nobel Laureate Adolph Butenandt managed to isolate the chemical responsible for the attraction of the female silkworm moth. He named this chemical bombykol for the genus of the silkworm moth, *Bombix mori* (Agosta, 1992; Wood, 1983).

Since the isolation of bombykol, it has been found that chemical signals are widespread in nature. They have been found in bacteria, fungi, algae, insects and other animals (Norris, 1980). Bacterial and fungal chemical signals are well defined and distinct. In the water mold *Allomyces* a single chemical, sirenin, functions as an attractant released by the female gamete which leads the male gamete along a chemotactic concentration gradient. This chemical is very specific. Minor changes in its structure obliterate its function. It induces a very distinct response, and it is effective at concentrations as low as 24 picograms per milliliter of water (Agosta, 1992). In more complex organisms the complexity of chemical signalling increases. For example, the sea anemone *Anthopleura elegantissima* emits chemical signals which influence the behavior of the whole target organism rather than just the gametes. The alarm pheromone, anthopleurine, produced by a wounded anemone induces a distinctive protective withdrawal reflex in its conspecific neighbors. It is also distributed by predator slugs who consume it but can't metabolize it. The anthopleurine is slowly excreted from the slug eliciting a protective withdrawal reflex in *A. elegantissima* anemones as the predator slug approaches (Agosta, 1992).

In mammals chemical signalling appears to be much more complex. This complexity has impeded the isolation of most mammalian pheromones. As indicated

above an early observation of chemical signaling in mammals was the attraction of a male dog to a bitch in heat induced by an as yet unidentified vaginal source, and the often observed marking behavior of dogs (Agosta, 1992). As with most mammals the chemical signal has not been identified, though not for lack of source material. It is estimated that four million liters of urine are produced annually by dogs in New York city alone (Agosta, 1992). Because of the complexity of mammalian responses to chemical signals (not to mention the complexity of the signal itself), progress in moving from an observed behavior to the elucidation of the chemistry and physiology involved has been slow. Isolation of a chemical and identification of it as a pheromone is dependent on the availability of an adequate bioassay that is specific and exhibits a response that is easily recognized by the investigator (Singer, 1991). Most of the current information regarding mammalian chemical communication is limited to behavioral observations, although in some instances the chemical identity of the pheromone has been established (Agosta, 1992). Many putative pheromones that have been isolated have little or no volatility and have a limited effective range. One such class of pheromones includes the deer lactones found in black-tailed deer urine. Close inspection and licking is required for transmission and results in recognition of individual animals. The male licks the urine and the lip-curl movements move the urine into the vomeronasal organ (VNO, also known as Jacobson's organ, see Figure 2) an organ believed to be involved in pheromone reception in many vertebrates (Agosta, 1992). The lip-curl (Flehmen) response of the male to female urine is well described and recognized as a pheromonal response. A similar response has been noted in cats, ungulates, bats, marsupials, and some others.

Figure 1. Structures of some representative pheromones. A, Anthopluerine from the sea anemone *A. elegantissima*, an ionic water born pheromone; B, bombykol from the silkworm moth, the first pheromone to be isolated and identified; C, deer lactone, a low volatility mammalian pheromone; D, the urinary pheromone in female house mice that delays puberty in other female mice, its six component chemicals are all necessary for full activity; E, androst-16-en-3-ol, one of the two steroids that make up the pheromone in boar saliva that induces lordosis in sows and has been marketed under the name "Boar Mate".

Many other pheromones are volatile and rely on airborne transmission. Few have been isolated but behavioral observations suggest their presence. One volatile pheromone that has been isolated is a boar salivary pheromone that induces lordosis (a receptive mating posture) in recipient sows. This pheromonal signal is composed of two steroids produced in the testes and stored in the salivary glands. This material has been produced and marketed as "Boar Mate" with great success for pig breeders. This is the first commercially recognized use of pheromones in mammals (Agosta, 1992). Most mammalian pheromones, however, have not been isolated and identified. Their existence has been determined strictly through physiological and behavioral observations.

II. Pheromones Defined

In 1959 the term pheromone was coined by the German biochemist Peter Karlson and the Swiss entomologist Martin Luscher to describe chemical signals between individuals of the same species. The term was derived from the Greek *pherein* (to transfer) and *hormone* (to excite) (Agosta, 1992; Albone, 1984; Beauchamp et al., 1976; Wood, 1983). E. O. Wilson (Wood, 1983) defined two classes of pheromones: (1) releaser (or signal) pheromones which induce immediate behavioral responses in the recipient such as attraction, recognition, or alarm and (2) primer pheromones which induce long term physiological (hormonal) responses in the recipient, such as reproductive maturation or alteration in reproductive status (Albone, 1984; Norris, 1980; Wood, 1983).

Chemical signalling also occurs between species. Such interspecific chemical signals are called allelomones and like pheromones have been divided into two classes: (1) allomones (allos + hormon - to excite others) which are chemical signals advantageous to

the emitter such as repellents, and (2) kairomones (kairos - opportunistic, exploitive) which are advantageous to the recipient such as attractants (Albone, 1984; Norris, 1980; Wood, 1983).

Specific conventions have been established based on insect studies to determine if a particular substance is a pheromone. The substance should have (1) a well defined behavioral or neuroendocrine function, (2) it should be unique in terms of its ability to induce a particular response, (3) it should be species specific, (4) the response of the recipient should have minimal dependence on learning, and (5) the signal itself should be composed of relatively few compounds (Beauchamp et al., 1976; McClintock, 1983a). It is important to note that many well established insect pheromones do not fit all five criteria (McClintock, 1983a). When mammalian pheromones are discussed this should be kept in mind, since there is much debate regarding terminology.

Most information concerning pheromones has been derived from insect studies. As in even simpler organisms, insects utilize relatively simple chemical compounds as chemical signals. Hundreds have been identified (Agosta, 1992). The usefulness of such natural signals in pest control has been a major factor motivating the study of insect pheromones. In addition, insects like simpler organisms, exhibit distinct responses to specific pheromones simplifying their elucidation. Recall that isolation of a chemical and its identification as a pheromone is dependent on the availability of an adequate bioassay. Sniffing, for instance, may not be an adequate bioassay, because for a pheromonal effect it is not necessary that the recipient be aware of an odor (Singer et al., 1987; Singer, 1991). Adolph Butenandt, when isolating bombykol, recognized that the male moth beat his wings in a "flutter dance" when exposed to an extract from the female moth that

contained the pheromone. With continued fractionation and the elimination of inactive extraneous substances, the extract was purified and the concentration required to elicit the response decreased. Eventually a chemically pure fraction that elicited the most intense response was identified and given the name bombykol (Agosta, 1992).

Understanding of mammalian pheromones is in its infancy and is limited due to the complexity of mammalian life and the mammalian brain (Agosta, 1992; Albone, 1984). Responses to pheromonal signals may not be as obvious, may be delayed, and most importantly, may be influenced by other incoming information and by learning (Agosta, 1992; Albone, 1984; Beauchamp et al., 1976). For example, mice have been shown to respond to chemical signals from stressed conspecifics (i.e., an alarm pheromone) but their response to the identical signal is different under various environmental conditions (Zalaquett and Thiessen, 1991). It is apparent that the response to the pheromone is dependent upon the endocrine status of the receiver at the time (Singer et al., 1987). The signals themselves may even be more complex in mammals, involving a combination of chemical compounds or signals (Agosta, 1992; Singer, 1991). Even insect pheromonal signals may be more complex than previously believed (Singer, 1991). Due to the signal complexity, Beauchamp et al. (1976) believes that the identification methodology of mammalian chemical signals needs to be reconsidered:

"In a species that has a complex vocal repertoire, it would be unlikely that a single note out of that repertoire carries all the information. It would also be improbable that if all the notes in the repertoire were taken and randomly emitted, the signal would remain whole any more than randomization of the notes of a familiar tune would leave the tune intact. In fact, looking at single notes or rearranging notes would only succeed in 'identifying' the components of the auditory signal if the signal were initially simple. Unfortunately these are exactly the

procedures employed in pheromone identification work. The search for *the* chemical may often be as fruitless as would be the search for *the* note of a song."

As such there is much discussion about the appropriateness of the term "pheromone" in mammalian chemical signalling. Many prefer the more generic term "social odor" (Agosta, 1992). Albone (1984) argues that the term "pheromone" is too precise to be used when discussing chemical signalling in mammals, due to the intricate combination of chemical and non-chemical signalling in these organisms. He prefers the term "semiochemical" meaning chemical sign or signal (Greek semion - sign) which includes within it the concept of pheromone. Beauchamp et al. (1976) argues that the term pheromone leads to unwarranted implications due to the criteria used to identify insect pheromones which are often unmet by mammalian putative pheromones. Singer (1991) argues that because many mammalian pheromones are associated with proteins and are transmitted by contact that they don't even qualify as a subclass of social odors. In addition, detectable odor is not a prerequisite for classification as a pheromone.

In order to avoid confusion, the following terminology will be utilized. The term "chemical signal" will be used to describe all classes of chemical communication both intra- and interspecific. This includes nonspecific olfactory communication. The term "pheromone" will be used to refer to a subclass of chemical signals when speaking of any chemical or combination of chemicals that results in a specific response within the same species. I do not believe, as the above investigators do, that the complexity of chemical signalling in mammals precludes the use of the term pheromone when a specific response can be demonstrated. The complexity of chemical signals in mammals means only that the elucidation of a single chemical that elicits the full specific response in the absence of other

cues may not be realistic, as Beauchamp et al. (1976) indicate.

The emphasis of this paper is estrus cycle primer pheromones which fit reasonably well most of the five criteria for a pheromone listed above (McClintock, 1983a). Thus, for the purposes of this report the terms chemical signal and pheromone will be consistently used where appropriate in order to avoid confusion resulting from alternative terminology.

III. Pheromones in Mammals

Most chemical signal research in rodents has been done utilizing mice. The pursuit of mammalian pheromones began in earnest shortly after the isolation of bombykol in the silkworm moth and the emergence of chemical communication as an accepted science. In 1959 it was recognized that social cues played a significant role in mouse, *Mus musculus*, reproductive behavior (Agosta, 1992). The importance of the olfactory system to mammalian communication in general, and in mammalian reproduction in particular is well established (Bronson and Macmillan, 1983). The best mammalian pheromone documentation is in rodents and is primarily mediated by receptors in the VNO. The response is abolished or significantly reduced when the VNO is ablated (Singer et al., 1987). In mammalian reproduction chemical signals appear to have three functions (1) dispersion (territory), (2) identification and attraction, and (3) triggering endocrine responses (primer effects) (Bronson and Macmillan, 1983). Many of the responses to pheromonal signals are well documented. The estrus cycle of the mouse is slowed by grouped females in the absence of a male, referred to as the Lee-Boot effect (Agosta, 1992; Norris, 1980; McClintock, 1981). In small groups the effect is to

suppress the estrus cycle while in large groups the result is complete anestrus or acyclicity (McClintock, 1981; Rogers and Beauchamp, 1976; Whitten, 1959). Puberty is also delayed by grouped females in the absence of a male. This delaying effect is mediated by compounds within the urine whose production is dependent on the adrenal gland and is detected by the VNO (Pandey, 1988; Vandenberg, 1986, 1989). Six compounds have been isolated and all six are required for the full effect (Agosta, 1992) further indicating the complexity of chemical communication in mammals (see Figure 1). The presence of a male has the opposite effects on the female, tending to accelerate the estrus cycle by inducing cyclicity in suppressed females (the Whitten effect) and advance the onset of puberty in immature females (the Vandenberg effect), while introduction of strange males will block implantation in newly impregnated females (the Bruce effect) (Agosta, 1992; Whitten, 1958; Vandenberg, 1967, 1969; Parkes and Bruce, 1962; McClintock, 1981; Norris, 1980). The puberty advancing effect of the male is an androgen dependent urinary pheromone that acts synergistically with tactile stimulation (Bronson and Macmillan, 1983). The Whitten effect is also mediated by an androgen dependent volatile urinary pheromone (Rogers and Beauchamp, 1976). Social signals that effect ovarian cyclicity in mice may function through variations in LH and prolactin secretion in the recipient (Bronson and Macmillan, 1983; Vandenberg, 1986).

Another rodent commonly used in pheromone studies is the Syrian golden hamster, *Mesocricetus auratus*. This species is particularly well suited for studies in chemical communication because of the high degree of dependence on olfaction and the VNO by the male for normal reproductive behavior. Such behavior is highly dependent on two chemical signals, a volatile attractant and a non-volatile copulation inducing pheromone

(Agosta, 1992; Nyby, 1983). The attractant pheromone that has been isolated from vaginal discharge is dimethyl disulfide (Agosta, 1992; Macrides et al., 1984; Nyby, 1983). This may not be the entire pheromonal signal since it alone does not induce as strong an effect as the vaginal discharge. All of the volatile components from the vaginal discharge may be required for the full effect (Agosta, 1992). The copulation inducing signal is a non-volatile water soluble protein (or an active molecule carried on the protein) in the vaginal discharge named aphrodisin that is delivered to the VNO by direct contact, namely licking (Agosta, 1992; Macrides et al., 1984; Singer et al., 1987).

There has been much commercial speculation about pheromones in humans, particularly by the perfume industry. Isolation of a human sex attractant would have significant marketability, but none have yet been identified despite the marketing of several perfumes that claim the attractive properties of pheromones. The pheromonal ingredient in these products is most often a pheromone identified and specific to domestic animals, such as Boar Mate (Bishop, 1992). While possibly providing a pleasing scent, this should not be confused with a pheromonal response (Berliner et al., 1991).

There is evidence of pheromonal communication in humans although those that have been observed are not "sex attractants." Receptors have been found on sperm which may respond to attractant chemicals released by the egg or nearby cells aiding in the migration of the sperm to the egg in the same fashion as the gamete migration in *Allomyces* (Agosta, 1992). Infants during the first six weeks post-partum have been observed to be able to identify their mother's breast versus other women's. This may be the result of chemical signals from the mother, marking chemicals from the infant, somatosensory

signals, or a combination (Agosta, 1992).

Several studies have indicated the presence of chemical signals that can alter the timing of the menstrual cycle in women. McClintock (1971) demonstrated that women living together soon exhibit menstrual synchrony, an effect recognized by many women. The author provided evidence indicating a pheromonal role over other possible interactions or cues such as a common photoperiod or inter-subject awareness. Russell (1980) demonstrated that axillary odors from a donor female could synchronize recipient females to the donors menstrual cycle. This was repeated and confirmed by Preti et al. (1986).

The possibility that male chemical signals may influence female reproductive cycling has also been investigated. The presence of men has been found to shorten the menstrual cycle in women. This is believed to be a pheromonal response (Veith et al., 1983). This was confirmed by exposing females to male axillary odors. The recipient's menstrual cycle was shortened and the variability reduced (Cutler et al. 1986).

IV. The Vomeronasal Organ (VNO)

Figure 2. The vomeronasal organ (VNO) also called Jacobson's organ.

From the preceding sections it is obvious that chemical signals / pheromones are

widespread in nature from the simple one celled organisms to mammals, including humans. Much work has been done to recognize and identify the structure of these chemical messengers and to determine the physiology of their action. The receptors for these chemical signals have been a focus of investigation. Both the vomeronasal organ (VNO) and the primary olfactory system (main olfactory bulb) have been implicated as the receptors for pheromonal signals. Supporting the concept of a primary olfactory system receptor is a study where female mice made peripherally anosmic by chemical means do not respond to an implantation protecting pheromone from stud males. Alternately, surgical ablation of the VNO in female mice does not abolish the spontaneous abortion blocking effect of stud males (Archunan and Dominic, 1990). These results demonstrate that the primary olfactory system must be intact for reception of this pheromone while the VNO need not remain intact. Much more research, however, has been directed at establishing the functional role of the VNO in pheromone reception. For many years it was believed that the VNO was not present or was vestigial in most mammals but it has since been found in virtually all vertebrate species, including humans (Garcia and Mondragon, 1991; Moran et al., 1991; Stensaas et al., 1991). The role of the VNO in the perception of pheromones and in the modulation of hormonal and behavioral responses in small mammals is now well established (Meredith, 1983; Wysocki and Lepri, 1991). In rats the LH release stimulated by the presence of males is reduced by ablation of the VNO (Rajendren et al., 1990). In mice, urinary priming pheromones from females in groups that inhibit sexual development or from males that accelerate the onset of puberty (Vandenbergh effect) apparently are detected by the VNO and induce changes in ovarian

function via changes in the hypothalamic-pituitary system (Vandenbergh, 1989). In humans the VNO was specifically stimulated by a putative human pheromone (ER-830) while standard odorants did not stimulate the VNO. The reverse was true for the olfactory epithelium (Monti-Bloch and Grosser, 1991).

Electrophysiological studies are just beginning to elucidate the functional pathway of the VNO. The VNO is directly connected to the accessory olfactory bulb located just behind the main olfactory bulb (Meredith, 1983; Brown, 1985). In rats and mice it has been demonstrated that there are projections from the accessory olfactory bulb to the amygdala with direct connections to the hypothalamus. These projections from the accessory olfactory bulb are localized to areas of the brain concerned with reproduction and social behavior. In contrast, projections from the primary olfactory bulb are more broad, extending to many areas of the brain (Meredith, 1991). This functional pathway can account for the reproductive effects associated with VNO chemoreception (Li et al., 1990; Meredith, 1991, 1983). Single compounds, which may be effective in stimulating the VNO, do not need to be volatile, but because they are transported through an aqueous phase it can be presumed that they must be water soluble (Singer et al., 1987). Both volatile and non-volatile water soluble compounds reach the VNO via mucus streaming (Meredith, 1991, 1983) and the receptors are responsive to both the volatile and non-volatile stimuli.

V. Physiology of Reproductive Primer Pheromones in Rats

The remainder of this report will focus on one aspect of regulation of the ovarian cycle, that due to social (pheromonal) interaction among females. In this study the rodent model used was the Sprague-Dawley rat, *Rattus norvegicus*, following the work of

McClintock and Adler (1978). In healthy lab rats the estrus cycle is usually four days in length (occasionally five). Proestrus is day one followed by estrus, metestrus, and diestrus. The estrus cycle can also be partitioned into three hormonally distinct phases (1) the follicular phase of the estrus cycle (defined as the period from the start of diestrus to the start of proestrus), (2) the ovulatory phase (defined as the period from the start of proestrus to the start of estrus), and (3) the luteal phase (defined as the period from the end of estrus to the start of diestrus). Ovulation typically occurs in early estrus. The timing of this cycle, and hence ovulation, can be altered by olfactory signals from other females in the social environment and has been demonstrated in many species. The modulation of the ovarian cycle by social (pheromonal) stimulation is widespread in mammals (McClintock and Adler, 1978). In humans the effect is to synchronize the menstrual cycle (McClintock, 1983a; Preti et al., 1986); in mice it is to lengthen or suppress the estrus cycle (Agosta, 1992; McClintock, 1983a). In the rat, like the human, chemical signals between females result in shortening and synchronization of their reproductive cycles (McClintock, 1983a, 1981). It has been found that grouped female rats (five or more) exhibit more regular cycles than isolated rats while the isolated rats exhibited cycles that were longer and more irregular (LeFevre and McClintock, 1991; McClintock, 1981). Within the period of three estrous cycles the cycles of the grouped individuals coincide (McClintock, 1981). This is different from the Whitten effect because it results from continuous interactions of ongoing cycles within the group rather than on an external signal, such as that from the male which induces simultaneous cyclicity in acyclic females (McClintock, 1981).

The makeup of the chemical signal that induces estrous synchrony in rats is unknown and the precise mechanism of action is poorly understood, as is true for the majority of mammalian chemical signals. It can, however, be deduced that the signal must be volatile since contact between individuals is not required for transmission. A common air supply is sufficient (McClintock and Adler, 1978; McClintock, 1981). McClintock (1981) argues that because the signal is airborne, not requiring physical contact between animals, it must be mediated by the primary olfactory system rather than the VNO. Never-the-less, as previously indicated, volatility of the chemical signal does not preclude the VNO as the receptor. Both volatile and non-volatile water soluble compounds have been demonstrated to stimulate the VNO (Meredith, 1983). In rats and mice the VNO may be a more logical receptor for a "primer" pheromone because it is directly connected to areas of the brain associated with reproduction. Thus the VNO would provide a direct route from pheromone receptor to areas of the brain associated with a "primer" response.

It is now apparent that there are two pheromonal signals, with opposing effects, that are emitted at different times during the rat's estrus cycle leading to estrus synchrony between rats. The first evidence for this was the demonstration that tonic odors from females in light-induced persistent estrus induced that same acyclic state in rats living downwind (McClintock and Adler, 1978; McClintock, 1981). Light-induced persistent estrus is a pathological acyclic state and may not function through the same mechanisms as chemical signals from normal phases of the estrus cycle that have not been modified by exogenous stimuli (McClintock, 1981, 1983a).

Pheromones emitted during the follicular phase of the estrus cycle shorten the estrus cycles of recipient females while pheromones emitted during the ovulatory phase lengthen

their cycles. Luteal phase odors apparently have no pheromonal component for they have no significant effect (McClintock, 1981, 1983a). The synchrony that occurs apparently is coordinated by the opposing effects of the two pheromonal signals (McClintock 1983a).

These two chemical signals were distinguished by exposing female rats to a constant airborne chemical signal from one of the three estrus cycle phases. The constant airborne chemical signals were generated by housing rats in the appropriate estrus cycle phase within an odor (chemical signal) source box. Each day the rats were replaced with rats entering the appropriate phase thus the odor source box always contained rats in a constant phase. Airborne chemical signals were carried to the experimental groups via unidirectional airflow. The estrus status of both the odor source group and the experimental groups were determined by vaginal cytology. The changes in vaginal cytology have been demonstrated to be good indicators of estrus cycle status and ovulation (McClintock, 1983a). During estrus the uterus swells, the vaginal epithelium becomes cornified, estrogen and LH rise leading to ovulation, and the rat exhibits a lordosis reflex on palpation (McClintock, 1981). Lordosis which is usually associated with the estrus cycle (strongest at proestrus/estrus) does not appear to be mediated through the same modality of social (pheromonal) signal as the estrus cycle. The two cycles become dissociated during social isolation and a shared air supply, which is sufficient to establish coordination of estrus cyclicity, had no effect on lordosis. The normal pattern of lordosis may depend on body contact (McClintock, 1981, 1983a). This separability of mechanisms which mediate sexual behavior (lordosis) and vaginal cytology implies different neuroendocrine mechanisms (Adler and Bell, 1969).

Two mechanisms are known to be involved in the timing of ovulation and consequently the length of the estrus cycle in the rat. One is the rate of growth of the follicle which is accelerated by FSH and retarded by progesterone. The other is the timing of the LH surge just prior to ovulation. Estrogen is required for the LH surge while progesterone can delay it (Hoffmann, 1973; McClintock, 1983a). Estrus cycle pheromones could act through any of these mechanisms to alter the estrus cycle so that synchrony might occur.

Vaginal cytology is useful in determining which mechanism is the most likely pheromonal route of control. Cornified epithelial cells are indicative of follicular estrogen while leucocytes are associated with progesterone from the corpus luteum (McClintock, 1983a). Shortening of the estrus cycle induced by follicular phase pheromones from diestrus females could occur through an acceleration of follicular development via FSH and resultant estrogen production which would induce the LH surge earlier. If this were the case there should be an increase in the number of three day cycles and in the number of cycles with a large portion of cornified cells during the follicular phase (McClintock, 1983a). Alternatively, follicular phase pheromones from diestrus females could shorten the estrus cycle by shortening the life span of the corpus luteum and reducing the amount of progesterone thus removing inhibition of the LH surge (reduced threshold) (McClintock, 1983a). This mechanism would not alter the length of the follicular phase. This mechanism where the life span of the corpus luteum is shortened is supported by observations of vaginal cytology (McClintock, 1983a). Follicular phase pheromones do not produce cycles with greater proportions of cornified cells, nor a greater number of three day cycles, and the follicular phase length is unaltered (McClintock, 1983a). This,

however, does not rule out alternative mechanisms, such as possible CNS stimulation or inhibition.

Ovulatory phase pheromones from proestrus females which lengthen the estrus cycle could modify estrus cycle timing through similar mechanisms (McClintock, 1983ab). They could delay the LH surge extending the follicular phase or extend the luteal phase by prolonging the life of the corpus luteum. The vaginal cytology of rats exposed to ovulatory pheromones exhibits extended periods of leucocytes indicating an extended luteal phase (McClintock, 1983a). This evidence indicates that estrus cycle pheromones most likely alter the length of the estrus cycle by modulating the life span of the corpus luteum and hence the length of the luteal phase rather than the length of the follicular phase (McClintock, 1983a).

The mechanism by which the life span of the corpus luteum is altered is unknown. Comparisons made between the effects of chemical signals emitted during different phases of the estrus cycle and different stages of pregnancy (i.e., pregnancy and lactation) suggest some possible similar neuroendocrine mechanisms (McClintock, 1983ab). The effect of chemical signals from pregnant females is comparable to the effect of chemical signals from diestrus (follicular phase) rats, in both cases the result is a shortening of the estrus cycle time course. Also in both of these reproductive conditions estrogen is rising suggesting that there could be an estrogen dependent mechanism leading to shortening of the estrus cycle (McClintock, 1983b).

Chemical signals from lactating females and their pups is comparable to the effect of ovulatory phase pheromones. In both lactating and ovulatory rats prolactin levels are

high, suggesting the possibility that lengthening of the estrus cycle could be prolactin dependent (McClintock, 1983b). In both situations, the suggestion of a similar mechanism is based entirely on a correlation between the effects of pheromones and the hormonal state of the animal providing the chemical signal. Such a correlation is more effective in ruling out possible mechanisms than it is in supporting them. Progesterone may not be the regulator of the corpus luteum's life span since progesterone levels are high in both pregnancy and lactation, which have opposite effects on cycle length (McClintock, 1983b). Also LH and FSH may be ruled out because both are low during pregnancy and lactation (McClintock, 1983b). The exact mechanism is as yet unknown, but the effects of pregnancy, lactation, and estrus cycle signals alter the length of the luteal phase by altering the life span of the corpus luteum and as such may function through the same neuroendocrine mechanisms (McClintock, 1983ab)

It is not yet known if synchrony is a result of a signal from a dominant female (a dominant signal) or if it is due to an interaction of equipotent signals from several individuals. The mechanisms discussed are conjecture based on known estrus cycle hormonal cycles and observed vaginal cytology.

Further supporting the hypothesis that estrus cycle pheromones alter cycle length by altering the life span of the corpus luteum comes from evidence of the timing of peak olfactory sensitivity. The rat is most sensitive to olfactory stimulation just prior to ovulation and consequently may also be most sensitive to pheromones at this time (McClintock, 1983a). This timing would be appropriate for altering the life span of the corpus luteum which forms immediately after ovulation. These mechanisms are different from those in mice. In mice the response to the pheromones released during each estrus

stage is different than in the rat and may be mediated via alterations in LH and prolactin secretion.

VI. Summary

Pheromonal physiology has a long and varied history and yet this discipline is in its infancy in regard to mammals. In mammals the study of pheromones is complicated by the complexity of mammalian life and the mammalian brain (Agosta, 1992; Albone, 1984). Responses to putative pheromones may vary with conditions and the pheromonal signal itself may be composed of a complex combination of chemicals. This situation has led to disagreement regarding the qualification of chemical signals in mammals as "pheromones." Furthermore, the terminology used is controversial.

In spite of these obstacles progress in the understanding of mammalian pheromones is ongoing and the rodent is the principal model. Both the primary olfactory bulb and the VNO have been identified as possible receptor organs for pheromones in mammals and general mechanisms for pheromonal effects have been hypothesized based on observed responses and known physiology.

In the rat two pheromones that act between females have been associated with the estrus cycle: follicular phase pheromones which shorten the recipient's estrus cycle, and ovulatory phase pheromones which lengthen the recipient's estrus cycle. The two alternating signals act to synchronize the cycles of grouped females most likely by altering the life span of the corpus luteum. The exact mechanism has not been determined. A third pheromone associated with the estrus phase that induces a state of persistent estrus has also been demonstrated.

The relative wealth of knowledge regarding such primer pheromones in female rats provides the opportunity for evaluation of pheromonal filtration by specially designed prototype filters for the Centrifuge Facility animal habitat on Space Station Freedom. Rats maintained in a state of light-induced persistent estrus have been demonstrated to emit an airborne pheromone which induces a similar state in recipient females in the absence of any other interaction (McClintock, 1978, 1984; McClintock and Alder, 1978). In this project this response is utilized in a bioassay to test the hypothesis that the filters are capable of blocking pheromonal transmission as well as other materials while withstanding a waste load over an extended period (up to 31 days).

A. PHEROMONE BIOASSAY STANDARDIZATION

MATERIALS AND METHODS

WIND TUNNEL CONFIGURATION. Two large enclosures or "housing units" were constructed from polyethylene storage boxes (29.5" x 19.5" x 15" , FDA approved for use in meat and poultry plants), plexiglass covers, and foam rubber weatherstripping (to create an airtight seal). A fan (1/125 HP, 60 CFM maximum capacity, Dayton model 4C440, Dayton MFG. CO., Chicago, IL), the two housing units, and the room exhaust vent were connected in series by 5' lengths of flexible aluminum ducting (4" I.D.). The configuration of the wind tunnel is illustrated in Figure 3.

The fan supplied the system's airflow, which was drawn from the colony room and vented directly out of the room's external exhaust vent. Therefore, there was no recirculation of air within the system. Airflow was controlled by a valve on the fan inlet.

The first or "upwind" housing unit consisted of 2 compartments each derived from a

polyethylene storage box. The upper compartment (compartment 1) served as a light-tight (aluminum foil lined) constant light source (24L:0D; 70 Lux, fluorescent, sunlight simulating spectrum, Vita-Lite, Duro-test corp., Fairfield, NJ). The lower compartment (compartment 2) was the actual animal housing space. The two compartments were

Figure 3. Exploded view diagram of the Wind Tunnel.

separated by a clear plexiglass plate which allowed light from the constant on light source to reach the animals below, while closing off the lower compartment and forming an airtight sealed chamber identical in all dimensions to the second or "downstream" housing unit. Airflow only entered and exited the bottom "animal housing" space. The downstream housing unit (compartment 3), ventilated by air coming from the upwind housing unit consisted of a single compartment derived from one polyethylene storage box

with a plexiglass plate cover. The downwind housing unit was exposed to colony room lighting. The clear plexiglass top allowed identical light cycle control within the housing unit as in the outside animal vivarium (12L:12D, 150-200 Lux, Lights ON 20:00-08:00). Timing of the rat estrus cycle is linked to the light-dark cycle. A 12L:12D lighting schedule results in almost exclusively four day cycles as opposed to 14L:10D or 16L:8D light cycles which result in a greater percentage of five day cycles (Hoffmann, 1968, 1973).

ANIMALS. Fifty female Sprague-Dawley rats (*Rattus norvegicus*) (Simonsen Laboratories, Inc., Gilroy, California) were divided into two groups: 34 were exposed to a normal light cycle (12L:12D, 150-200 Lux, fluorescent, Lights ON 20:00-08:00) and 16 were exposed to constant light (24L:0D; 70 Lux, fluorescent sunlight simulating spectrum, Vita-Lite, Duro-Test Corp., Fairfield, NJ). Ten rats approximately 140-165 days of age (235-250g) were selected from the colony of 34 normally cycling rats based on the regularity of the length of their estrus cycles around 4 to 5 days. These rats were randomly divided between two large polypropylene (20" x 16" x 8") "shoebox" type rat cages, forming two groups of five rats. Group one was placed in the downwind housing unit (compartment 3) of the wind tunnel (experimental group); group two was housed with the colony and served as a vivarium housed control group. An additional (3rd) group of five rats was selected from the 16 rats kept in constant light for 30 days prior to their selection. Exposure to the constant light induced a state of persistent estrus (light induced persistent estrus). These rats were placed in the upwind constant lighted housing unit (compartment 2) of the wind tunnel and served as an airborne chemical signal

(pheromone) source, i.e., the constant light odor source group. Presumably, the state of persistent estrus resulted in a constant pheromonal message. This constant pheromonal message has been demonstrated to induce the same acyclic state in rats living downwind (McClintock and Adler, 1978; McClintock, 1981). The induction of persistent estrus is relatively rapid and increases with age and light intensity (Hoffmann, 1973; Lawton and Schwartz, 1967). The follicle develops to the preovulatory phase believed to be mediated by the pineal gland, allowing increased release of gonadotropins and a resultant increased continuous level of estrogen with resultant vaginal cornification (Fiske et al., 1960; Hardy, 1970; Hoffmann, 1973; Ifft, 1962). These rats were maintained in constant light (24L:0D) for the duration of the experiment.

Standard rat chow (Wayne® Rodent BLOX® 8604, Teklad Premier Laboratory Diets, a Harlan Sprague Dawley Inc., Co., Madison, WI) and water were provided ad libitum. Temperature was maintained at $25\pm 3^{\circ}\text{C}$. Pine chip bedding was changed weekly. The plexiglass top of each box was sealed (air tight) with foam rubber weatherstripping and held together with six 3 inch spring clamps (allowing for daily access to the animals).

PROCEDURE. Beginning fifteen days prior to the start of this study the estrus cycle of each rat was monitored via analysis of vaginal smear cytology (Hoar and Hickman, 1983). Vaginal smears were collected daily (2-4 hours after lights off) under dim red light using isotonic saline moistened microtip cotton swabs. Dim red light is equivalent to a "night time" condition in that it does not influence the circadian rhythm of the rat (Lynch, 1988). Slides were dried and stained with Wrights/Giemsa stain (VWR Stat Stain, VWR Scientific Inc., Brisbane, CA). Red light was provided with a 15 watt

incandescent bulb and a red filter (50% cut-off at 660 nm, 4% transmission, Roscolux RoscoSun #27 medium red, Musson Theatrical, Santa Clara, CA). Estrus typically occurs in the dark between subjective noon and midnight (Hoffmann, 1973; Young et al., 1941). All handling was carried out during the dark period both to facilitate recording the estrus phase and to avoid interfering with the rats during their inactive period. Rats were weighed daily and water consumption for the group was measured. During handling, animals were assessed for health following criteria set by the San Jose State University Campus Veterinarian. Each rat group was handled separately to avoid artificial transmission of pheromones and a 70% non-denatured ethanol wash was used to clean hands and all materials that the rats might be exposed to during handling. Rats were housed in the wind tunnel apparatus beginning on study day 16. Access was provided by removing the plexiglass cover of the housing units. This required that the fan and light source (upwind housing unit) be turned off briefly while the units were open. Rats in the constant light odor source group (upwind housing unit) were exposed briefly to the dark as they were moved to an adjoining lighted room where their vaginal smears were collected.

During Experiment A-1 of this study the airflow through the wind tunnel was maintained at 22 CFM for a period of 24 days thus allowing for airborne signal interactions between the upwind constant light odor source group and the downwind experimental group. This allowed for about 4-5 estrus cycles.

During Experiment A-2, the airflow was reduced to 10-12 CFM through the system and the study was extended for 21 additional days. All other aspects of the experiment

remained the same as in Experiment A-1.

Vaginal smears were scored for percent of each of the three typical cell types found (nucleated epithelial, cornified epithelial, and leucocyte). Diestrus is represented by a preponderance of leucocytes, proestrus by mostly nucleated epithelial cells, estrus by mostly cornified epithelial cells (some nucleated), and metestrus by an even mix of cornified and nucleated epithelial cells (possibly some leucocytes) (Adler and Bell, 1969; Hardy, 1970; Hoar and Hickmann, 1983; Young et al., 1941). Rats were then rated as either "in estrus" or "not in estrus" based on the percentage of cornified epithelial cells present in the vaginal smear. Rats were rated as "in estrus" only if 80% or more of the cells present were cornified epithelial cells. Three investigators were involved in scoring the prepared slides. Random scorings of previously read slides confirmed the uniformity of scoring across investigators. The lordosis reflex was not measured as was done in previously published work (McClintock and Adler, 1978) due to the separate nature of the mechanisms which mediate the lordosis reflex and vaginal cytology (McClintock, 1981, 1983a).

STATISTICS. Because we were interested in the dependence of estrus status (as defined by an estrus / not-estrus scoring system) on the hypothesized presence or absence of pheromones, a test for independence was most appropriate. The number of rats determined to be in estrus each day during the ten day statistical sampling period were counted as were rats that were not in estrus. These estrus / not-estrus counts were compared between control and experimental groups and between airflow conditions using Chi-Square analysis. Ratings of "in estrus" and "not in estrus" results in data that fell under the category of enumeration data, that is, data that results from counting units in

different groups (Scheffler, 1979). This kind of data limits the type of statistical analysis that may be applied. The Chi-Square test for independence was selected because all assumptions for this test were met by our data.

RESULTS - Pheromone Bioassay Standardization

Chi-Square analysis of estrus status for both control and experimental rats across all airflow conditions indicates that estrus status was not dependent on either condition, control or experimental, or high (days 30-39) or low (days 51-60) airflow ($p < .25$). Visual inspection of the data (see Figures 3 and 4) suggests that a smaller percentage of the experimental rats were in estrus as compared to controls, particularly during the low airflow condition. Each condition was further analyzed independently.

Chi-Square analysis of estrus pattern in the downwind (experimental) rats during the final ten days (days 30-39) of the twenty-four day high airflow (22 CFM) period in the wind tunnel revealed no significant effect of airborne signals from the upwind source group ($p < .50$). We did not replicate the results of McClintock and Adler (1978). The final ten days (days 51-60) of the twenty-one day low airflow (10-12 CFM) period in the wind tunnel revealed a more pronounced decrease in estrus frequency of the experimental "downwind" rats as can be seen in Figures 4 and 5. The effect is in the opposite direction of what would be predicted based on previously published results (McClintock and Adler, 1978; McClintock, 1984). The apparent effect is not significant ($p > .10$) so that we cannot conclusively say that it is dependent on pheromones present in the low airflow, i.e., in our study fewer animals were in estrus compared to the control group.

Comparisons between the estrus status of the experimental rats versus high and low airflow conditions reveal no significant effect of flow rate on the estrus status of the experimental rats ($p < .50$). Because of the apparent difference in the percentage of rats exposed to constant light that were in estrus (see Figure 5) during the high and low airflow conditions this difference was tested post hoc (Tukey test for comparison between means) (Broota, 1989). The difference was found to be significant ($p < .05$). Under both flow conditions the estrus status of the rats exposed to constant light is significantly different from the control rats ($p < .001$).

Table 1. Daily Estrus Percentages (Bioassay Standardization, Experiments A-1 & A-2)

Day	Control group	Const. Light group	Experimental group	Day	Control group	Const. Light group	Experimental group
1	40	40	75	31	40	100	20
2	40	60	80	32	60	60	20
3	0	60	20	33	40	80	60
4	60	60	40	34	40	40	50
5	60	80	0	35	40	80	20
6	50	80	80	36	60	80	20
7	40	80	60	37	20	80	0
8	100	75	20	38	0	60	20
9	30	80	0	39	20	80	25
10	20	40	0	40	20	80	0
11	0	100	100	41	40	100	0
12	20	80	60	42	40	80	0
13	60	60	0	43	0	60	60
14	80	20	40	44	20	80	0
15	80	20	60	45	60	100	40
16	80	100	60	46	20	80	40
17	40	100	60	47	40	80	40
18	40	60	0	48	60	80	40
19	20	20	60	49	0	80	20
20	20	40	20	50	20	100	0
21	60	100	40	51	20	100	0
22	40	80	60	52	60	80	40
23	40	60	60	53		80	20
24	20	100	0	54		80	20
25	60	80	60	55		100	20
26	40	20	20	56		100	0
27	20	20	0	57		80	20
28	20	40	40	58		80	20
29	0	20	40	59		80	0
30	40	40	20	60		80	20

Note: Bold type indicates periods used for statistical analysis. Control rats (n=5, housed in the animal vivarium) were placed in group cages on day 16. Data from days 26-35 were used for control group statistical analysis. Constant light pheromone source group rats (n=5, to be housed in compartment 2) and experimental rats (n=5, to be housed in compartment 3) were placed in group cages on day 16. For both groups data from days 30-39 were used for high airflow sampling (22 CFM, Experiment A-1), the airflow was reduced to 10-12 CFM on day 40, and days 51-60 were used for low airflow sampling (Experiment A-2).

Figure 4. Daily Estrus Percentages (Bioassay Standardization, Experiments A-1 & A-2). These plots illustrate the percentage of rats in estrus each day based on vaginal smear cytology. (A) Control group (n=5) housed in the rat vivarium and exposed to background colony chemical signals (rats were placed in group cages on day 16, statistical analysis was performed on data from days 26-35), (B) the chemical signal source group (n=5, exposed to constant light in compartment 2) and, (C) the experimental group (n=5, compartment 3) exposed to air containing chemical signals from the chemical signal source group (placed in group cages in the wind tunnel on day 16, days 30-39 were used for high airflow sampling (Experiment A-1), on day 40 airflow was reduced to 10-12 CFM, and days 51-60 were used for low airflow sampling (Experiment A-2)).

Figure 5. Total Percentage of Rats in Estrus (Bioassay Standardization, Experiments A-1 & A-2). The bar graph compares the total percentage (mean $\pm$ S.E.M.) of rats in estrus over the ten day sampling periods for the control, odor source (constant light - persistent-estrus), and the experimental groups under both high and low airflow conditions.

B. PHEROMONE FILTERING BIOASSAY / FILTER PERFORMANCE

MATERIALS AND METHODS

PROTOTYPE FILTER. The filter is a 16" x 13.25" x 1.9" panel type High Efficiency Particulate Air (HEPA) filter. Multiple layers of materials are designed to provide a wide range of filtering capabilities from low molecular weight chemicals (i.e. pheromones) to large debris such as animal dander and feces. The side of the filter exposed to animal waste is composed of a highly absorbent wastepad (designed to accommodate the waste load of six rats) capable of withstanding a high solid waste load, absorbing urine, water and other liquids while maintaining acceptable airflow parameters throughout its service life. The filter also incorporates layers of bacterial retentive media,

immobilized activated charcoal and other odor absorbers, and is impregnated with phosphoric acid to neutralize ammonia (Strength et al., 1993). The precise makeup of the filters is Pall Corporation (Glen Cove, Long Island, NY) proprietary and is not reported here.

BIOISOLATION TEST FIXTURE. The biofiltration test fixture (BTF) is a self-contained, air tight "wind tunnel" designed by McDonnell Douglas Space Systems Company (Huntington Beach, CA) with provisions for measurement of air pressure across the filters, air flow, and other air quality parameters. Two identical test units were fabricated and used for this study. Each unit consists of a "group habitat" (13" x 16" x 8"), a plenum and mixing chamber, air ducts, and 6 odor/acoustic isolation cages (each 19" x 10" x 8") in styrofoam insulated containers. The BTF group habitats are constructed of 1/2" acrylic, with a stainless steel feeder and group habitat floor and Teflon hoses. Figure 6 illustrates the different components that make up a single BTF.

During live animal testing, air was continuously pumped unidirectionally through the inlet filter, group habitat, outlet filter, and plenum, and the air was equally distributed to 6 isolation cages. Pressure transducers with a range of 0.23-3.0 inches of water (Ashcroft Model XLdp) provided a continuous measure of pressure drop across the inlet and outlet filters. Flow through the test fixture was continuously measured using a fixed-orifice flowmeter in the air supply line. Pressure drop and flow readings were stored on an IBM Personal Computer using a multi-channel data acquisition card (Strawberry Tree Computers, Inc., Sunnyvale, CA). Access to the interior of the habitat was available through an air tight door. Air samples were collected from group habitat and plenum air

sampling ports.

Figure 6. Exploded view diagram of the Biofiltration Test Fixture (BTF).

Each group habitat contained six female rats and each isolation cage housed a single female rat. Food (1 gram rodent grain dustless precision pellets, BioServ, Frenchtown, NJ) and water were available to all rats ad libitum. Food and water were provided in the group habitat via a bulk storage feeding tray designed to reduce food dust and two "lixit"

water valves, respectively.

A single fan (3/4 HP, Howell Electric Motors, Plainfield, NJ) was used to induce airflow through both test fixtures. The inlet air was drawn from the test room and the outlet air from the isolation cages was exhausted directly to the outside of the building. In this way there was no recirculation of air through the system. Airflow was maintained in the test fixture at 22 CFM as measured by a fixed-orifice flowmeter via a butterfly valve.

The insulated containers containing the single isolation cages had clear tops so that the light-dark cycle in each cage would be that of the experimental room (12L:12D, 90-130 Lux, lights ON 20:00-08:00, fluorescent sunlight simulating spectrum, Vita-Lite, Duro-test Corp., Fairfield, NJ). The BTF group habitat was isolated from room light by an outer light isolation box and exposed to constant light (24L:0D, 100 watt incandescent) within the box. Light intensity ranged from 50 to 250 Lux across the floor of the habitat. The majority of the floorspace was under light intensities of approximately 130-140 Lux.

Test fixture A (BTF A) had filters at both the inlet and outlet ports of the group habitat while test fixture B (BTF B) had no outlet filter. Therefore, BTF B served as a control for the unobstructed transmission of pheromones. Experiment B-1 lasted 21 days once the rats were placed in the BTFs.

In Experiment B-2 (a repeat of Experiment B-1 with no rats in the isolation cages) both the inlet and outlet filters were in place in both BTFs. The light isolation box around the group habitat was removed and the outlet air was vented directly to the outside of the building. The group habitat was exposed to constant room lighting (24L:0D, fluorescent) with an average intensity of about 200 Lux over the majority of the floor space.

Experiment B-2 lasted 31 days once the rats were placed in the BTFs.

ANIMALS. In Experiment B-1 twelve female Sprague-Dawley rats (*Rattus norvegicus*) approximately 200-225 days of age (245-275g) were selected from the remaining colony of normally cycling rats (12L:12D, 150-200 Lux, fluorescent, Lights ON 20:00-08:00) based on the regularity of the length of their estrus cycles around 4 to 5 days. These rats were then split between the isolation cages of the two test fixtures, forming two groups of six singly housed rats of similar makeup. An additional group of twelve rats was selected from the remaining colony of rats kept in constant light (24L:0D, 70 Lux, fluorescent sunlight simulating spectrum) for 79 days prior to their selection. Exposure to the constant light induced a state of persistent estrus (light induced persistent estrus). These rats were placed in the group habitats of the test fixture and served as an airborne signal (pheromone) source, the state of persistent estrus presumably resulting in a constant pheromonal message. These rats were exposed to light (24L:0D) for the duration of the experiment.

In Experiment B-2 twelve female Sprague-Dawley rats (*Rattus norvegicus*) approximately 225-250 days of age (approximately 245-275g) were selected from a new colony of rats kept in constant light (24L:0D, 70 Lux, fluorescent, sunlight simulating spectrum) and placed in the group habitats of the BTFs. These rats were exposed to room light (24L:0D) for the duration of the experiment.

PROCEDURE.

Pheromone Filtration Bioassay. Experiment B-1. Starting fifty days prior to placing the rats in the test fixtures the estrus cycle of each rat was monitored via analysis of vaginal smear cytology. Vaginal smears were collected daily (2-4 hours after lights off)

under dim red light. Dim red light is equivalent to a "night time" condition in that it does not influence the circadian rhythm of the rat (Lynch, 1988). Red light was provided with a 15 watt incandescent bulb and a red filter (50% cut-off at 660 nm, 4% transmission, Roscolux RoscoSun #27 medium red, Musson Theatrical, Santa Clara, CA).

Following initial animal placement in the BTFs, the singly housed experimental rats housed in the isolation cages were weighed and water consumption was measured on alternate days. Vaginal smears were collected every day. During handling, animals were assessed for health following criteria set by the San Jose State University Campus Veterinarian. Each rat was handled separately to avoid artificial transmission of pheromones and a 70% non-denatured ethanol wash was used to clean hands and all materials to which the rats might be exposed during handling. Rats housed in the group habitats were removed for weighing, vaginal smear collection, health assessment, and water consumption measurements on alternate days. Rats in the isolation cages were protected from light exposure by draping a light opaque cover over the isolation cages when accessing the group housed rats in constant light. All handling was carried out during the dark period both to facilitate recording the estrus phase and to avoid interfering with the rats during their inactive period.

Vaginal smears were scored for percent of each of the three typical cell types found (nucleated epithelial, cornified epithelial, and leucocyte). Rats were then rated as either "in estrus" or "not in estrus" based on the percentage of cornified epithelial cells present in the vaginal smear. Rats were rated as "in estrus" only if 80% or more of the cells present were cornified epithelial cells.

In Experiment B-2 animals were placed only in the group habitat of the BTFs and

were not removed from the BTFs for the duration of the experiment. All measures of filter performance made in Experiment B-1 were measured again except for pheromone transmission and odor transmission.

Ammonia Analysis. Ammonia measurements were made from the habitats lower air sampling port and the downstream mixing plenum on alternate days during both Experiment B-1 and B-2 (Sensidyne Gastec 1-30 ppm detector tubes (#3L) and calibrated pump (kit #800)). Prior to leaving the room each day, all hose connections were checked and airflow was adjusted as needed.

GC/MS Gas Analysis. Air samples were collected on the last day of Experiment B-1 (day 71) and both midway through and at the end of Experiment B-2 (days 18 and 27-29) and shipped to the Perkin-Elmer Corporation (Pomona, CA) for gas analysis by a Perkin-Elmer chemist. Samples were collected by drawing air from the biofiltration test fixture (or ultra pure helium (99.997%) tank in the case of the "blank", American Welding Supply, San Jose, CA) through a transport line (Teflon tubing), a U-tube containing Tenax (referred to as a Tenax trap), an additional connecting line (Teflon), and a 24 VDC air pump (supplied by Perkin-Elmer). The Teflon tubing was attached to the Tenax traps via Valco unions and air from the pump was discharged directly into the room. All traps were supplied sealed in plastic bags purged with ultra pure helium. Immediately prior to sampling the transport line and Valco union were purged with ultra pure helium (99.997%) for at least 15 minutes. The Tenax trap to be used was then removed from its bag and the caps on the trap removed and placed in a clean sealable plastic bag. The trap was then attached to the pump and BTF air sampling ports via the Teflon connecting lines

and Valco unions and the samples were collected over timed intervals. During sampling air flow from the pump was measured for assessment of sample volume. Volumes ranged from 200ml to 30L. Immediately following sampling the trap was capped and sealed in a plastic bag purged with ultra pure helium for overnight shipment to Perkin-Elmer where the GC/MS analysis was done. At Perkin-Elmer the samples were thermally offgassed into a gas chromatography column and the components were detected and identified using a mass spectrometer set to scan for typical contaminants in waste gas between 34 and 250 atomic mass units (Strength, 1993). The transport line was purged with ultra pure helium for at least 10 minutes between each sample.

Microbial Analysis. Midway through Experiment B-2 (day 22) air samples were drawn from the BTFs through sterile 0.22 μm Millipore filter paper in a sterilized holder using a vacuum pump (27 in. Hg). Air samples were drawn for 45 and 90 minutes from both the habitat and the outlet plenum. The Millipore filters were sealed in a sterile container and sent to Perkin-Elmer for microbial analysis.

Odor Assessment (sniff) Test. The protocol for the odor assessment was modified from the procedures described in NASA Handbook #NHB 8060.1C (1991) and only performed at the end of Experiment B-1, on day 67. Following a protocol approval by the SJSU Human Subjects-Institutional Review Board, sniff test subjects were recruited from the SJSU student population and surrounding community via posted fliers. Subjects were collectively briefed on their participation on the day of the sniff test assessment, and were free to ask questions about their participation. Subjects were then examined by a San Jose State Health Center Physician (mouth, pharynx, and nasal passages) prior to the odor assessment. Subjects were again examined by the physician for signs of irritation

following the odor assessment.

Following orientation and medical evaluation, subjects were taken singly into the odor assessment room (a room which had no detectable background odor as determined in a pretest). Subjects were directed to assess the relative strength of nine odor standards presented as 10 ml of clear fluid within individual 60 x 20mm petri dishes. The odor standards used are defined in Table 2. Emphasis was placed on scoring the relative strength of the odor standards and not the odors appeal (pleasantness, unpleasantness). Odors were scored by participants on a scale of 0-4: 0 = undetectable, 1 = barely detectable, 2 = easily detectable, 3 = objectionable, 4 = revolting. To qualify as a test subject the person had to be able to distinguish between 7 primary odor standards and 2 water blanks.

Table 2. Odor assessment odor standards (modified from NASA Handbook #NHB 8060.1C, 1991)

Odor	Chemical Composition	Dilution
ether	diethyl ether	135ul/500ml
camphor	1,8-cineole	80ul/500ml
musk	15-hydroxypentadecanoic acid lactone	0.5mg/500ml
blank	distilled water	-
floral	rose water	7ml/500ml
mint	menthone	3ul/500ml
pungent	glacial acetic acid	1.5ml/500ml
putrid	methyl disulfide	5ul/500ml
blank	distilled water	-

Three additional odor samples were then presented to the subjects in the form of 50 cc air volumes dispensed from glass syringes directly in front of their noses. The 50 cc

air samples were drawn from 1.6 liter Tedlar® gas sampling bags (Cole-Parmer Instrument Co., Chicago, IL) containing a volume of air collected from a) the test fixture animal habitat, b) the outlet mixing plenum (i.e., filtered habitat air), and c) ambient air drawn from the study room as a control. A score of 1.5 or less was established as acceptable by NASA (NASA, 1992).

STATISTICS. Statistical analysis was performed only on data from the pheromone filtering bioassay and the odor assessment. The remaining assays did not require statistical analysis. As in the pheromone bioassay standardization portion of this project the Chi-Square test for independence was used to assess the relationship between estrus status and the hypothesized presence (or absence) of pheromones.

In the human panel odor assessment we were interested in the relationship between the odor source (control versus samples) and the odor scores. Specifically, we wanted to know if there was any difference between the average odor score for the control and the experimental samples. A t-test is adequate for individually comparing each samples average score to the control or an analysis of variance can be used to compare all combinations of average odor scores (Broota, 1989). Either method is valid and both were used for this analysis.

RESULTS - Pheromone Filtration Bioassay / Filter performance

Pheromone Filtration Bioassay. Experiment B-1. Chi-Square analysis of the dependence of estrus status of singly housed experimental rats on the presence or absence of a filter (i.e., hypothetical presence or absence of pheromones) and on the sampling condition (control period days 41-50 or experimental period days 62-71) does not indicate

that estrus status is dependent on either condition ($p < .75$). Individual Chi-Square analysis of estrus status versus each condition also indicates that they are independent. Chi-Square evaluation of the estrus status of the singly housed experimental rats in each test fixture versus the presence of a filter between the group habitat and the isolated rats was not significant ($p < .75$). Chi square analysis also found no significant difference in estrus status in either group between the control period and the experimental period after exposure to air theoretically containing chemical signals ($p < .5$). A reduction in the percent of rats in estrus between the unfiltered and filtered conditions is noticeable in Figures 7 and 8. A difference is also evident in the percent in estrus for the filtered group before and after the filter is installed (i.e., between the control and experimental periods). In both cases the difference is not statistically significant.

Ammonia Analysis. No ammonia was detected in either the habitat or the plenum of either test fixture during Experiments B-1 or B-2.(i.e., all measurements were less than 0.5 ppm).

GC/MS Gas Analysis. Gas chromatography / mass spectrophotometry (GC/MS) performed by Perkin-Elmer on air samples collected during both Experiments B-1 and B-2 found no measurable waste gases from either the animal chamber or the mixing plenum.

Microbial Analysis. Measures made of microbial content in the air drawn from the BTFs during Experiment B-2 (using a sterilized filter as a trap for microbes) were at last report inconclusive.

Odor Assessment (Sniff) Test. Experiment B-1. A frequency histogram of averaged odor scores is presented. Air samples from both the habitat and the plenum were

found to be either undetectable or barely detectable. In both cases the average odor score was below the barely detectable threshold and well below the criterion average score of 1.5 set for passing, the same as the ambient room air control. Statistical analysis using both the t-test and analysis of variance indicates that the averaged odor scores for the three odor sources are not significantly different ($p < .75$).

Filter Loading. The pressure drop across the outlet filter of test fixture A during Experiment B-1 was linear (from 0.54 inches of water to 0.82 inches of water) over the course of 22 full days of testing (see Figure 10). There was no overt indication of filter failure during this test. Slight adjustments in the airflow regulating valve were made during the last few days of the study to maintain the flow rate; no other adjustments were needed. It is noteworthy that upon completion of this test, the waste material on the filter appeared dried and, in many places, almost resinous in appearance (similar to dried tree sap). Three-hundred ninety-five grams (395g) of desiccated waste material was removed from the filter at the end of the Experiment B-1 (1 day after removal of the rats).

In Experiment B-2 it was discovered that the inlet filters were lighter by 45.8 to 66.7 grams, respectively, at the end of 31 days. This suggests that the high airflow may have had a desiccating effect. The outlet filters were heavier after waste removal by 115.4 to 128.5 grams, respectively, at the end of the same time period. This increase in weight is most likely due in part to the inability to remove waste embedded in the filter screen. Waste material that was effectively filtered and trapped within the filter undoubtedly makes up a part of this weight gain. The pressure drop across the outlet filters of both test fixtures was relatively linear (from about 0.5 inches of water to about 1.1 inches of water) over the course of 31 full days of testing (see Figure 11). There was no indication of filter

failure during this test. Slight adjustments in the airflow regulating valve were made to maintain the flow rate; no other adjustments were needed.

Table 3. Daily Estrus Percentages (Pheromone Filtering Bioassay / Filter Performance)

Day	Isolated (Expt.) BTF A	BTF B	Constant Light BTF A	BTF B	Day	Isolated (Expt.) BTF A	BTF B	Constant Light BTF A	BTF B
1	33.3	83.3	66.7	83.3	37	50	33.3	100	100
2	100	100	83.3	83.3	38	16.7	0	83.3	100
3	33.3	33.3	66.7	83.3	39	16.7	50	83.3	100
4	50	16.7	50	16.7	40	16.7	16.7	100	100
5	16.7	50	66.7	33.3	41	20	16.7	100	100
6	33.3	50	66.7	83.3	42	16.7	16.7	83.3	100
7	100	60	83.3	100	43	33.3	0	100	100
8	33.3	16.7	83.3	83.3	44	40	66.7	100	100
9	33.3	16.7	83.3	100	45	0	33.3	100	100
10	60	50	83.3	50	46	50	16.7	100	100
11	0	0	83.3	83.3	47	33.3	16.7	100	100
12	33.3	40	83.3	66.7	48	16.7	0	100	100
13	16.7	33.3	83.3	33.3	49	40	50	100	100
14	16.7	50	66.7	66.7	50	20	0	100	100
15	33.3	33.3	100	83.3	51	16.7	0	100	100
16	20	0	100	100	52	20	33.3	100	100
17	66.7	0	100	100	53	50	50	100	100
18	16.7	33.3	83.3	100	54	0	16.7		
19	33.3	66.7	83.3	83.3	55	33.	0	100	100
20	16.7	50	100	100	56	33.3	40		
21	20	0	83.3	100	57	33.3	16.7	100	100
22	33.3	16.7	100	100	58	0	50		
23	0	0	100	100	59	0	16.7	100	100
24	0	33.3	100	100	60	16.7	16.7		
25	16.7	50	100	100	61	0	33.3	100	100
26	0	0	100	83.3	62	16.7	33.3		
27	33.3	33.3	100	100	63	33.3	33.3	100	100
28	16.7	16.7	100	100	64	0	0		
29	0	0	100	100	65	16.7	50	100	100
30	50	66.7	100	100	66	16.7	0		
31	0	50	100	100	67	16.7	66.7	100	100
32	33.3	16.7	100	100	68	16.7	33.3		
33	16.7	33.3	100	100	69	33.3	16.7	100	100
34	16.7	0	100	100	70	16.7	0		
35	33.3	33.3	100	83.3	71	16.7	50	100	100
36	0	0	83.3	100					

Note: Bold type indicates periods used for statistical analysis. Data from days 41-50 (prior to placement in the BTF) were used for control period statistical analysis. Data from days 62-71 were used for experimental period statistical analysis following placement in the BTF on day 51. BTF A has a filter present between the chemical signal source rats (Constant light exposure) and the isolated experimental rats while BTF B does not.

Table 4. Odor Score Frequencies (Pheromone Filtering Bioassay / Filter Performance).

Odor	Score:				
	undetectable	barely detectable	easily detectable	objectionable	revolting
Ether	0	1	6	1	0
Camphor	1	1	6	0	0
Musk	2	4	1	0	1
Blank	7	1	0	0	0
Floral	0	0	5	2	1
Mint	0	1	5	1	1
Pungent	0	1	3	4	0
Putrid	0	2	2	3	1
Blank	7	1	0	0	0
Habitat	4	4	0	0	0
Plenum	3	5	0	0	0
Room Air	5	3	0	0	0

Note: n=8.

Figure 7. Daily Estrus Percentages (Pheromone Filtering Bioassay / Filter Performance). This plot illustrates the percentage of rats in estrus each day based on vaginal smear cytology for each rat group: (A) both group housed and isolated experimental rats housed in BTF-A in which a filter is present between the chemical signal source rats and the experimental recipients, (B) both group housed and isolated experimental rats housed in BTF-B in which no filter is present between the chemical signal source rats and the experimental recipients and thus serves as a control for filtering performance. Data from days 41-50 (prior to placement in the BTF) were used for control period statistical analysis. Data from days 62-71 were used for experimental period statistical analysis following placement into the BTF on day 51.

Figure 8. Total Percentages of Rats in Estrus (Pheromone Filtering Bioassay / Filter Performance). This bar graph illustrates the total percentage (mean $\pm$ S.E.M.) of rats in estrus over the ten day sampling periods before and after placement in the BTF's for the four groups of rats.

Figure 9. Average Odor Scores (Pheromone Filtering Bioassay / Filter Performance). Averaged odor scores for odor standards and air samples from both the habitat and the plenum of BTF-A are illustrated. Scores are on a scaled of 0-4: 0 = undetectable, 1 = barely detectable, 2 = easily detectable, 3 = objectionable, 4 = revolting. Air sample scores are well below the criterion average value of 1.5 set for passing (Strength et al., 1993).

Figure 10. Filter Air Flows and Pressure Differentials (Experiment B-1). These plots illustrate (A) pressure drops and (B) the average airflows (set at 22 CFM) across both inlet and outlet filters within each biofiltration test fixture. Rats were placed in the BTF on day 51.

Figure 11. Filter Air Flows and Pressure Differentials (Experiment B-2).

These plots illustrate (A) pressure drops and (B) the average airflows (set at 22 CFM) across both inlet and outlet filters within each biofiltration test fixture. Rats were placed in the BTF on day 1.

DISCUSSION

Previously, studies have indicated that airborne chemical signals (pheromones) from rats in a state of light induced persistent estrus induce a similar state of persistent estrus in rats exposed to these airborne chemical signals (McClintock and Adler, 1978). During an 18 day exposure period to airborne chemical signals the percentage of rats that were in estrus rose to 83% (averaging 56%) which was well above the average of 25% in estrus within the control group (McClintock and Adler, 1978).

This response was utilized in a bioassay in order to test the chemical signal filtering efficiency of prototype filters designed for use in the orbital rodent habitat on the Centrifuge Facility of Space Station Freedom. The initial bioassay standardization procedure using a wind tunnel designed and built at San Jose State University (Figure 5) did not show a significant effect of airborne chemical signals on the downwind group (25% in estrus as compared to the vivarium control group at 35%, see Table 1 and Figures 4 & 5). The persistent-estrus chemical signal source group in this study were in estrus 70% during this period, lower than the previously published results of 80% (McClintock and Adler, 1978). It is possible that this lower percentage of rats that are in estrus within the source group (70%) may play a part in the absence of a significant observed pheromonal effect. This difference may be effective, when coupled with a high airflow and unknown airflow distribution patterns within the wind tunnel, in masking the response. The high airflow may have been sufficient for diluting any airborne signal beyond an effective threshold concentration. Airflow patterns may have contributed to this if the airflows did not provide sufficient mixing time within the animal cage, particularly with the animal bedding in the upwind odor source cage which may have

served as a reservoir for volatile chemical signals.

When the airflow through the wind tunnel was reduced by half to test for a possible airflow related effect, a change in the status of the recipient group was recorded (Table 1, Figures 4 & 5). During this period the source group had 86% in estrus, significantly higher than during the high flow condition, while the recipient group was in estrus less frequently (down to 16%) when compared to the vivarium control group (35%, see Figure 4). This apparent effect is not statistically significant. However, the trend is opposite to what would be expected based on previously published results. It is possible that the signal from the constant light persistent-estrus group may include as a component of the signal the previously described ovulatory phase pheromone which is known to lengthen the estrus cycle, thus reducing the frequency of occurrence of the estrus stage, possibly by extending the lifespan of the corpus luteum (McClintock, 1983ab). Perhaps the different signals recognized as being associated with the different estrus cycle phases are not distinct pheromones but varying combinations of several cyclically secreted pheromones in a fashion similar to the cyclically secreted reproductive hormones. Any variable that may alter the proportion of each signal that is delivered in a combination of signals will alter the effect. In this case it is possible that the message received more closely resembled the message associated with the ovulatory phase resulting in the observed decrease in estrus counts. This is, of course, conjecture based on the trend in the data.

Recall also that, in mammals, responses may be influenced by other incoming sensory information and by learning (Agosta, 1992; Albone, 1984; Beauchamp et al., 1976) and that the status of the recipient at the time the signal is received can result in a

different response (Singer et al., 1987; Zalaquett and Thiessen, 1991). Such complexity of responses requires a well defined bioassay. This standardization bioassay did not control for all possible confounding variables which may have played a role in the observed effect, for instance, the previously mentioned unknown airflow patterns. There was no control for auditory communication between the persistent estrus group and the recipient group nor the colony itself, noise levels including fan noise were not measured and, the materials used to construct the wind tunnel also were untested for possible offgassing.

When repeating this bioassay using the biofiltration test fixture (BTF) provided by McDonnell Douglas Space Systems Company (Figure 6), which provided better control, no significant effects of airborne chemical signals were observed (Table 3, Figures 7 & 8). In the unfiltered group the percent of rats in estrus increased from 22% during the control period to 29% during the experimental period in the BTF while the presence of a filter resulted in a change from 27% during the control period to 19% during the experimental period in the BTF as shown in Figure 8. Although these changes were observed, they are not statistically significant for such a small sample size, indicating that no chemical signal (pheromonal) effect was demonstrated and thus no conclusion can be made about the filter's pheromone filtration properties. The lack of a significant effect where one would be expected may be a consequence of low pheromone sensitivity in the rats selected. The breeding protocol used by most animal suppliers selects for rats with "normal" 4-5 day estrus cycles. This may also inadvertently select for rats with a reduced sensitivity to primer pheromones (McClintock, 1992).

Although the results are not statistically significant, some observations may be

relevant. The observed trend in the data, if real, suggests that an effect may be present but that it may simply not be great enough to be significant with such a small sample size. The lack of a demonstrable effect may, in turn, be the result of dilution of the chemical signal by the high airflow designed into the system. If real, the slight increase in estrus percentage (from control levels) in the unfiltered group (from 27% to 29%, Figure 8) follows the expected direction of change that would be expected for exposure to chemical signals from light induced persistent-estrus rats according to previously published results (McClintock and Adler, 1978). On the other hand, the slight decrease in estrus percentage (from control levels of 27% down to 19%, Figure 8) for the rats exposed to filtered air may be a result of their isolation. Recall that isolated rats (isolated from all modes of communication) exhibit longer and more irregular cycles than rats in groups of five or more (LeFevre and McClintock, 1991; McClintock, 1981). If indeed all pheromonal signals are effectively filtered it would be expected that the downwind recipient rats which are housed singly would exhibit characteristics typical of rats completely isolated from other rats, such as longer cycles represented by a lower estrus percentage. Although these results are suggestive, they are not statistically significant and must not be mistaken for a supported conclusion.

The difference in the observations when using the BTF versus the SJSU wind tunnel may stem from differences in the control of confounding variables. McDonnell Douglas Space Systems Company designed the BTFs and had them tested for auditory isolation between each isolated experimental animal and between the isolated animals and the group housed animals, for offgassing of materials used to construct the BTFs, and for

airflow patterns. The airflow patterns may actually have been too uniform in some regards as will be indicated below. At San Jose State further measures were taken to ensure the adequate and uniform lighting for all animals and elimination of unacceptable fan noise. The greater effort made to control extraneous variables may account for the different observations made in the low flow standardization bioassay and this pheromone filtering bioassay.

The additional measures of filtering efficiency, other than the waste loading capacity (feces, dander, and urine) of the filters are more suggestive of effective filtering. The lack of detectable ammonia or waste gases in either the habitat or the mixing plenum suggest either extremely effective filtering and airflow parameters that obscure air sample analysis. The lack of a difference between the air samples drawn from the animal habitat and the filtered habitat air from the plenum make it difficult to quantify filter efficiency. The "cleanliness" of the air samples probably came about due to two factors. The uniform airflow through the BTF may be sufficiently unidirectional so as to prevent any backflow of air within the habitat from the animal waste or the animals themselves. If this is indeed the case all of the air sampled from the animal habitat would be comprised of essentially HEPA filtered room air uncontaminated by animals or animal wastes downstream of the air sampling port. If this is the case it can be concluded that the filters are exceptionally efficient since air sampled from the plenum which has passed around the animals and their accumulated waste (feces, dander...) before passing through the filter, is equally free of contaminants. The other possibility is that the airflow is high enough that any animal odor or chemical signal is effectively diluted beyond detectable concentrations for the assay used and if so no conclusion regarding filter efficiency can be drawn. I believe, however,

that both factors contribute to the obtained results, and that the lack of detectable gases downstream of the outlet filter is due to dilution effects and effective filtering of materials that although present in the animal habitat were not detected. As mentioned previously the observation of a slight shift in the percentage of estrus, possibly due to pheromonal effects, although not statistically significant, if real suggest that animal products are present and are filtered. The sensitivity of the rat to diluted pheromones may be greater than the sensitivity of our instruments.

The same airflow parameters are probably responsible for the low odor assessment scores for all air samples. No significant difference is observed between habitat and plenum air, both having an average odor score below the barely detectible level and only slightly more detectible than room air (Table 4, Figure 9). Such small differences for odors that are already at the threshold of sensibility are particularly insignificant and may again be a result of the high air flows resulting in dilution of the odorant chemicals. Whether the filter is effectively filtering out detectible odors cannot be definitively determined since no odor could be detected prior to filtration. It is significant to note, however, that no odor was detected and this is a highly desirable result.

Of all the tests performed on these filters the most significant results came from the evaluation of the waste loading capacity of the filter. The filter exceeded the 14 day required lifetime by at least 8 days (Experiment B-1) and 17 days (Experiment B-2) exhibiting only a linear increase in pressure drop as waste built up over the 22 (Experiment B-1) or 31 (Experiment B-2) day experimental period. This is clearly shown in Figures 10 and 11. Again the high airflow may have played a role. It is noteworthy

that the waste removed at the end of the study period was desiccated, suggesting that the high airflow served as a drying agent preventing excess fluid accumulation. If present, fluid build up could have resulted in obstruction of the filter by forming a wet waste and fluid barrier to airflow.

CONCLUSION

The results of this project are very encouraging. The waste loading capacity of these filters far exceeded the minimum goal of 14 days showing no overt sign of failure after 31 days. Ammonia, odors, and other airborne contaminants were not detected in filtered outlet air, suggesting effective filtration. There is some suggestion that pheromones may be filtered as well. In the more controlled biofiltration bioassay an effect similar to previously published results is suggested, although the results are not statistically significant. The lack of statistical significance may not be a reflection of what was being tested but may be due to (1) reduced pheromonal sensitivity of the rats selected for this experiment, (2) high airflow which may dilute airborne signals below their optimal effective concentration, and (3) a small sample size which demands a large observed effect for statistical significance. Further study is necessary before a positive conclusion can be made about pheromone filtering efficiency.

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