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THE INFLUENCE OF THE MAJOR HISTOCOMPATIBILITY COMPLEX, GUT BACTERIA AND DIET IN THE PRODUCTION OF ODOURS OF INDIVIDUALITY IN RODENTS

by

HEATHER MACINTOSH SCHELLINCK

Submitted in partial fulfillment of the requirements for the degree of Ph.D

at

DALHOUSIE UNIVERSITY

HALIFAX, NOVA SCOTIA

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ABSTRACT

Individual animals can be identified by their unique urine odours. To determine the influence of genetic, microbiological and dietary factors in producing urinary odours of individuality in rodents, a series of experiments was conducted using two different paradigms: a habituation-dishabituation task and a go/no go operant task. Results revealed that rats and mice with genetic differences at the major histocompatibility complex have urine odours which can be discriminated by conspecifics. The presence of gut bacteria influences the production of these urine odours. The removal of different types of bacteria changes the nature of the urine odour; however, the specific nature of the odour-producing bacteria could not be determined. Rats did not respond to probes of urine odours from conspecifics whose bacteria had been selectively depleted even though further testing in a discrimination task revealed that some aspects of these odours were familiar. Mice on different diets produce urine odours which are easier for rats to discriminate than urine odours from mice of different MHC genotypes. The discrimination of urine odours from MHC congenic rats is disrupted by changes in diet whereas the discrimination of urine odours from rats on the same diet is not disrupted when the urine odours are changed to those from rats of a different MHC congenic strain. These results indicate that odours of individuality are multidimensional, involving genetic, dietary and bacterial factors. When different dietary cues are available, they may be more influential than genetic factors in providing discriminable odour cues.

LIST OF ABBREVIATIONS AND SYMBOLS USED IN THE TEXT

cm	centimeter
EOG	electro-olfactogram
GC-MS	gas chromatography-mass spectrometry
GI	gastrointestinal
gm	gram
id	inside diameter
kg	kilogram
L:D	light dark cycle
l	liter
MHC	major histocompatibility complex
mg	milligram
ml	milliliter
mm	millimeter
N	number
od	outside diameter
id	inside diametert

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Burt Slotnick willingly provided the expertise necessary to construct the operant-olfactometer as well as the software required to run the system. Many of my experiments would never have materialized without his assistance. Gordon Troop was also very helpful in offering me many practical hints as to how to keep my olfactometer running smoothly.

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My husband lost his hair and my children grew up during the course of my PhD. Without their support and patience, it would have been difficult for me to enjoy myself in the Department of Psychology. They accepted without question that running rats had become the most important part of my life.

CHAPTER 1

EMPIRICAL EVIDENCE FOR ODOURS OF INDIVIDUALITY
PART 1: INVERTEBRATES AND VERTEBRATES
EXCLUDING THE *RODENTIA*

The ability of animals to recognize each other as individuals is vital for effective social interactions. Mate recognition, parent-offspring interactions and the identification of kin depend upon successful use of cues to discriminate between individual conspecifics. Recognition cues may be visual, auditory or olfactory nature and in some instances, cues from more than one modality may act in concert (Beecher, 1989). The relative importance of each sensory modality likely depends upon the perceptual specialization and ecological niche of individual species. In birds, auditory cues appear to be most relevant to the recognition process (Beecher & Stoddard, 1990); in non-primate mammals and in invertebrates olfactory-based cues appear to predominate (Blaustein, Beckoff, & Daniels, 1987; Wilson, 1987). In this thesis, the focus is upon recognition by olfactory cues.

Chapter 1 is devoted to an overview of the literature which illustrates the role of olfactory stimuli in the production of individual recognition cues. Section one examines the problems in defining individual recognition. In sections two and three, experimental studies that have demonstrated odour-mediated recognition of individual invertebrates and vertebrates other than rodents is presented. The purpose of such a review is to illustrate the range of species and variety of contexts in which different olfactory cues may be used to signal individuality.

1.1. Toward a definition of individual recognition

The definition of individual recognition has not always been consistent. Many investigators have considered the ability to discriminate between odours from two different animals to be equivalent to individual recognition (Halpin, 1986). In contrast, Barrows, Bell and Michener (1975) have stated that the ability to discriminate between subgroups of odours (e.g., previously encountered odours versus novel odours or odours associated with positive versus negative experiences) should not be considered individual recognition. They consider that individual recognition should be defined as "a learned discrimination among conspecific individuals" (p. 2824). This argument suggests that even though an olfactory signal may be unique, if the receiver does not associate this uniqueness with a particular conspecific, then individual recognition does not occur.

Beecher (1989) has defined individual recognition as varying from "simple discrimination of one or a few individuals (e.g. offspring) from all other individuals, to discrimination of each individual in the group from every other individual" (p. 249). He goes on to say, however, that from the perspective of examining cues, the requirements are the same. For example, if a number of unrelated offspring are in one nest, every mother must be able to discriminate her own young among the many available. Beecher states that "the requirement for the signature system that any individual in the group be distinguishable from all others is equivalent to the requirement that each individual in the group be distinguishable from every other" (p. 250).

The focus of this thesis is upon the information emitted by the sender rather than the mechanism by which the receiver comes to recognize the signal. Consequently, I will adopt Beecher's more liberal view and review the evidence for individual recognition by

odours in parent-offspring interactions, mate choice and differential interactions between kin and non-kin.

I am interested in the source of odours of individuality. It has been suggested that constant cues of a genetic origin, as well as more variable environmental factors such as those provided by the nest environment and diet, may interact to produce an odour profile unique to each individual (Halpin, 1986). In addition, physiological changes which reflect an animal's age, sex, emotional state and reproductive status may influence the production of individual odours (Brown, 1979). It is the purpose of this thesis to further investigate the origin of olfactory cues which mediate individual discrimination in rodents. In particular, the role of the major histocompatibility complex (MHC) and microbiological and dietary factors in producing odours of individuality in rats and mice will be investigated.

1.2. Olfactory recognition cues in invertebrates

One might expect that given the vast number of invertebrate species, there would be a wealth of knowledge regarding odour discrimination processes. With the exception of the extensive literature on kin recognition in insects and the recent interest in allorecognition in sea squirts, however, this does not seem to be the case. Only sporadic reports of olfactory communication in such a diversity of species as sea anemones (*Anthopleura elegantissima*)[1] and American lobster (*Homarus americanus*) are available (Francis, 1973; Atema, 1986; Ayre, 1987). This dearth of information appears to reflect an overall lack of investigation rather than a lack of olfactory recognition on the part of invertebrates.

---

[1] The Latin name of each species discussed will be included in brackets after the common name the first time the species is introduced. Thereafter, the common name only will be included.

For example, an extraordinary amount of information has been collected from the one species of marine crustacean in which the role of olfactory cues in individual recognition of conspecifics has been thoroughly examined. The stomatopod (*Gonodactylus*) can discriminate between the odours of two previously encountered opponents, learn olfactory information regarding the sex and size of an opponent and possibly remember the odours of mates for at least two weeks (Caldwell, 1985, 1987, 1992). It seems likely that olfactory recognition cues will be discovered in many other invertebrates as well. The issues to be investigated and the kind of information to be gained are exemplified by research on the odour-mediated behaviour of the seasquirt (*Botryllus schlosseri*) and several species of Insects (*Insecta*).

1.2.1. Seasquirt *(Botryllus schlosseri)*

The seasquirt is made up of individual zooids which share the same blood supply and associate within a common tunic. It has been determined that the swimming larvae of the seasquirt fuse with genetically similar individuals to form a colony. The preference for relatives could be simply a result of limited dispersal patterns or it could be mediated by some form of self versus non-self discrimination. Scofield, Schlumpberger, West and Weisman (1982) reported that fusion in seasquirts is controlled by a gene which shows a degree of polymorphism similar to that of the vertebrate MHC (See Chapter 3 for a discussion of the MHC and polymorphism). Subsequently, Grosberg and Quinn (1986) developed a mathematical model of the relationship among dispersal patterns and genotypes in this species. They determined that limited dispersal could not account for the spatial association of identical siblings from the same colony. They also were able to show that *Botryllus* larvae introduced to an established colony associated with individuals of the same histocompatibility type regardless of the similarity of their background genes. This

experiment provides the most convincing evidence to date that recognition of kin is linked to histocompatibility type. It has been speculated that heterozygosity at the histocompatibility loci in the seasquirt is promoted by outbreeding (Scofield et al., 1982; Grosberg, 1987) but this hypothesis has not been tested experimentally.

1.2.2. *Insecta*

The ability of insects to discriminate between the odours of conspecifics has been studied in the contexts of mate selection, queen recognition and discrimination of nest mates. As nestmates are frequently related to one another, discrimination between nestmates and non-nestmates is frequently referred to as kin recognition. Nest odours may be derived genetically from each individual or the colony queen, from the environment or possibly from a "Gestalt" odour mixture produced by all of the colony members (Carlin & Holldobler, 1986). Among the species most extensively studied are the fruit fly (*Drosophila*), the sweat bee (*Lasioglossum zephyrum*), the wasps (*Polistes*), the honey bee (*Apis mellifera*) and the ants (*Formicidae*). The following synopsis of odour-mediated recognition behaviour in these species highlights some of the most significant findings in this area.

1.2.2(a) Fruit flies (*Drosophila*): Mate selection in fruit flies appears to be the result of female response to male courtship activity. While visual, auditory and tactile cues are relevant to choice behaviour (Spieth, 1974), olfactory cues appear to predominate. When compared with wildtype females, mutants that were "smell-blind" could not discriminate between different strains of males, virgin or experienced males or males of two different ages (Markow, 1986).

Gas chromatographic analyses of volatiles from different strains of fruit flies revealed that there were quantitative variations in male pheromones and that males with the most pheromone mated most frequently (van den Berg, Thomas, Hendriks, & van Delden, 1984). It has also been suggested that quantitative differences in female pheromones can influence courtship behaviour in males. After determining that a specific hydrocarbon (7, 11-heptacosadiene) induced wing vibration, a discrete step in male courtship, Jallon and his colleagues (Antony & Jallon, 1982; Jallon, 1984) determined that females of one strain were preferred by males of their own and other strains. A positive correlation was found between the duration of wing vibration and amount of heptacosadiene. The ability of males or females to discriminate between opposite sex conspecifics of the same strain based upon quantitative differences in heptacosadiene or other hydrocarbons has not been tested. If such a discrimination could be made, it could form the basis for testing for assortative mating in fruit flies.

1.2.2(b) Sweat bees (*Lasioglossum zephyrum*): Both male and female sweat bees discriminate between the odours of individual females. Analysis of male mating behaviour has shown that male sweat bees discriminate between females or their odours in both a laboratory setting and in the field. In a habituation-dishabituation task, the mating behaviour of males to an odour-impregnated paper dishabituated in response to the odour of a novel female (Barrows, 1975). In the field, different populations of males were attracted to different females which had been previously killed by freezing but which still retained their odour characteristics (Michener & Smith, 1987). Mate selection by males appears to be a result of discrimination of genetically distinctive odours. Across the seven degrees of relationships examined, a negative correlation was found between the attempts

of a male to mate with a second female and her relationship with the first female (Smith, 1983).

A gas chromatography-mass spectrometry (GC-MS) analysis revealed that the Dufour's gland of female sweat bees contained a mixture of four lactones which could produce mating responses in males (Smith, Carlson & Frazier, 1985). This mixture elicited greater responses from males than any of the individual compounds. Furthermore, principal components analysis of pheromone samples which had been extracted by a gas chromotographic headspace analysis showed that ratios of lactones are more similar among nestmates (Michener & Smith, 1987). In a follow-up study, Smith and Wenzel (1988) determined that nestmates are closely related and that relatedness covaries with respect to the pheromonal products of the Dufour's gland. The genetic locus which controls the production of these compounds has not been identified, nonetheless, this report appears to be the first to describe a correlation between specific olfactory recognition cues and genetic similarity.

The interaction between guard bees and their conspecifics at burrow entrances has been examined in female sweat bees. Female guards block the narrow entryway and generally only let residents enter. In lab tests, the presence or absence of this behaviour can be used to measure recognition (Greenberg, 1988). Although living bees were generally used in these experiments, guards also respond to moist paper taken from an intruder's nest or to bees killed by freezing. Such results have been interpreted to mean that odour-mediated cues are used for individual discrimination (Bell, 1974; Barrows, Bell, & Michener, 1975).

Female guards admit nestmates or intruders which are most closely related to themselves (Bell, 1974; Barrows et al., 1975; Greenberg, 1979). This behaviour has been interpreted to mean that recognition has a genetic component. Further proof for this

hypothesis comes from an examination of the influence of nest odours on worker recognition. Nest odours do not seem to mask a worker's individual odour. Bees raised in colonies of related and unrelated individuals were rejected by the sisters of their unrelated nestmates which were raised in different nests but were accepted by their own sisters from another colony (Buckle & Greenberg, 1981).

The nature of discriminable cues also appears to be influenced by the age of the females and their diet. Young adult females are more likely to be accepted into a nest than older females (Bell, Breed, Richards, & Michener, 1974); guard bees on a mixed diet will not accept a bee raised on only one component of the diet (Greenberg, 1981 as cited in Greenberg, 1988). Despite the assertion that genetic cues are predominant (Mchener & Smith, 1987), it appears that at least female sweat bees make use of additional cues from the environment to discriminate among individuals.

1.2.2(c) Wasps (*Polistes*): The ability to discriminate between individuals in wasps has generally been investigated in females of the species. The few studies which have examined mate selection by males took place in the lab and no evidence of discrimination behaviour was found (Post & Jeanne, 1983). Female workers discriminate between nestmates and non-nestmates and most studies have demonstrated that environmental cues arising from nest odours provide the critical recognition cues. Females spent more time with their sisters only if they had been previously exposed to the sister's nest odours; if the subjects were exposed to sisters without their odours or if the subjects had no experience with nest odours, they did not discriminate between nest and non-nestmates (Shellman & Gamboa, 1982). Moreover, if the nests of two potential queens were exchanged prior to eclosion, the workers did not discriminate between their actual sisters and their pseudosisters (Pfennig, Reeve, & Shellman, 1983).

Further support for the role of nest odours in producing discriminable individual cues, comes from a laboratory study in which the behavioural interactions of females were examined in a neutral arena (Venkataraman, Swarnalatha, Nair, & Gadagkar, 1988). In this context, nestmates and non-nestmates could be discriminated but only by wasps which had been exposed to their respective natal nest and nestmates. Thus, compared with sweat bees, odours of recognition in wasps generally seem to be mediated by an environmental component. Nonetheless, potential queens seem to detect a genetic component in recognition cues as they do not discriminate between nestmate sisters and non-nestmate nieces (Gamboa, Klahn, Parman, & Ryan, 1987). The possibility that the queen uses different cues than the workers to discriminate individuals has not been explored.

The nest paper of wasps has been analyzed in an effort to determine the composition of the characteristic colony odour. This paper contains the same hydrocarbons which are present in the cuticle of the colony members. Moreover, when the hydrocarbons were removed from two nests, workers could not discriminate between their own nest and that of another colony (Espelie, Wenzel, & Chang, 1990). Further study has revealed that workers did not recognize individuals from nests which had their hydrocarbons removed four days prior to the test; nestmates from untreated nests were recognized (Singer & Espelie, 1992). Such results confirm that surface hydrocarbons play a significant role in the recognition of nestmates.

1.2.2(d) Honey bees (*Apis mellifera*:): Honey bees appear to use both environmental and genetic cues to recognize queens and identify nest members (Breed & Bennet, 1987). For example, swarming queenless bees are able to discriminate between their own queen and an unfamiliar queen (Boch & Morse, 1974; Boch & Morse, 1982). Breed (1981) held cues originating from water, food and genetic variation in the workers constant to

demonstrate that genetic cues originating with the queen were responsible for the discrimination. He found that sisters of the queen were preferred to non-sister queens.

Other characteristics of the queen which may contribute to her uniqueness include variations in her age and reproductive status. Bees from a dequeened swarm preferred to associate with a mated queen of the same age as their original queen rather than a virgin queen but preferred an old virgin rather than a young one (Ambrose, Morse, & Boch, 1979). The queen's mandibular gland pheromone complex (QMP) appears to produce the majority of the behavioural activity in the colony members (Slessor, Kaminski, King, & Winston, 1990).

The importance of dietary cues for discrimination among honey bee colonies was stressed by Kalmus and Ribbands (1952). First, they demonstrated that foragers were attracted to the food source that their nestmates had been trained to visit; then, they showed that after a colony was split in half, naive workers would visit the same dish associated with the trained workers in their half of the colony. The authors hypothesized that food odours enabled colony members to identify one another.

To examine the influence of genetic cues on colony discrimination in honey bees, Breed (1983) removed eggs from two different hives and transferred them to a third hive for rearing. Pupae were removed prior to emerging. Individuals from these groups were accepted by related groups but not by unrelated groups of bees. This result provides convincing evidence for the use of genetic-based cues for discrimination of colony members in bees

In addition to learning genetic cues, bees may learn a composite group odour. Workers raised in mixed sister:non-sister groups did not respond aggressively to sisters or non-sisters raised in isolation (Breed, Butler, & Stiller, 1985). Breed and his colleagues suggested that this result supports the hypothesis that worker bees learn a composite

identity of the group. This may, however, reflect the ability of bees to use differential genetic information as recognition cues. The introduced isolated non-sister workers which were accepted into the nest could not have had any association with colony odours but they could have a genetically derived odour similar to that of their sisters who had been in the nest. The finding in this same experiment that, overall, the bees interacted preferentially with their previously unknown kin rather than their non-sisters, provides further evidence for kin discrimination.

To assess the relative importance of genetic and environmental cues for individual recognition, Breed, Stiller and Moor (1988) examined the development of odour recognition in bees in laboratory and field settings. They determined that as soon as day-old bees from lab groups were exposed to foreign group odours for only 30 minutes, they were not accepted into lab groups of sister bees. Both related and unrelated bees were accepted into lab groups when they were introduced immediately after emergence but, by the time the bees were 12 hours old, only sisters had a high probability of being accepted. While this finding emphasizes the importance of kin related cues, it should be noted that regardless of relatedness, no bees of 3 days of age or older were accepted. Thus, it would appear that the hive environment does generate olfactory recognition cues. These colony odours could be produced by the queen, be related to diet, a general blend of nestmate odours or some combination of these factors.

Comb wax, which is the main nesting material of honey bees, has been identified as one source of recognition cues. Moreover, bees maintained separately on the same comb as unrelated bees were accepted into the latter's colony at the same rate as sisters (Breed, Williams, & Fewell, 1988). Thus, it would appear that environmental cues can mask genetic cues in some instances.

Based upon an examination of bees' response to odours from comb wax, two hypotheses related to recognition of nestmate odours have been developed, the "common feature model" and the "proportion hypothesis". The common feature model was derived from the analysis of a bee's response to two compounds derived from comb cues separately and in combination (Breed & Julian, 1992). Donor and recipient groups of bees were raised with one of the two odours, hexadecane (H) and methyl docosanoate (M), or the odours in combination and the behavioural interactions among the groups analyzed. Most interesting was the finding that bees raised in the presence of H were not rejected when introduced into a combination of H and M but those raised in the presence of M were rejected in the combination. Breed & Julian (1992) suggested that this finding supports the common feature model of nestmate recognition in bees with the presence or absence of a particular cue being of critical importance.

This latter interpretation was criticized by Getz (1993) who suggested that the data are consistent with a "proportion hypothesis" in which a mixture takes on the odour of a salient component. Breed (1993) counteracted Getz (1993) hypothesis by providing data that showed that changes in the ratio of the compounds in the wax did not influence the choice behaviour of the bees. It may be that different cues provide context-dependent information such that cues which are ignored during an aggressive encounter are relevant for another behaviour such as queen recognition.

An investigation of the neural mechanisms activated during processing of an odour may help resolve the issue of the genetic versus environmental basis of odour-mediated recognition cues in honey bees. Presently, techniques for recording from olfactory receptor neurons and the mushroom bodies during olfactory processing are being perfected (Stengl, 1994; Schafer, Rosenboom, & Menzel, 1994). The first *in vivo* recordings from Kenyon cell interneurons of mushroom bodies during olfactory processing have recently

been made in locusts (Laurnet & Naraghi, 1994). As the mushroom bodies are involved in learning and memory, it may be possible to analyze the ability to discriminate different odours using this procedure.

1.2.5(e) Ants (*Formicidae*): In ants, behavioural bioassays recording affiliative behaviour (huddling and food exchange) and aggression (grabbing and stinging) are used to assess recognition and discrimination of individuals (Carlin & Holldobler, 1986). Most of the research which has examined the source of recognition cues in ants has focused upon the importance of odours produced by the queen although the role of nestmate odours and cues originating from diet have also been evaluated.

In several different species of ants, workers from a queenless colony were accepted by a neighbouring colony whereas those reared with a queen were not (Schneirla, 1971; Holldobler & Taylor, 1983; Jaffe & Marcuse, 1983). Moreover, in carpenter ants (*Camponotus*) when unfamiliar sisters were on different diets they were accepted as long as they had been reared with the same queen (Carlin & Holldobler, 1986). Nonetheless, odour cues are also contributed by nestmates. For example, queenless carpenter ants on the same diet were less aggressive toward one another than unrelated workers and sisters raised with different queens were less aggressive than non-sisters in the same situations (Carlin & Holldobler, 1986).

In other instances, diet-related cues appear to predominate. Former nestmates of leaf cutting ants (*Acromyrmex octospinosus*) reared on different diets displayed high levels of aggression whereas unrelated workers on the same diet were not aggressive (Jutsum, Saunders, & Cherrett, 1979). In carpenter ants, although queenless workers were less aggressive toward their unknown relatives than to non-kin, the levels of aggression toward kin increased when their diet was different (Carlin & Holldobler, 1986). In fire ants

(*Solenopsis invicta* Buren), raising unrelated colonies on the same diet reduced levels of aggression compared with that in groups raised on different diets (Obin & Vander Meer, 1989). Moreover, the addition of alien queens to resident and intruder colonies for 28 days did not increase levels of aggression, an indication that dietary cues may predominate in some species. Further investigation revealed that adding only one unique component to a diet was sufficient to increase the level of aggression toward an individual. Removing a component also affected recognition cues, in some instances (Obin & Vander Meer, 1989).

The availability of odour recognition cues may also be affected by the age of individuals, their nest experience and reproductive status. Carpenter ant workers which were less than one day old were more readily accepted into alien colonies than five day old workers. In addition, workers which were taken from their own colony when they were less than 12 hours old were less acceptable to their nestmates than were workers who had been in the nest for five days prior to removal (Morel, Vander Meer, & Lavine, 1988). In a species of ants (*Rhytidoponera confusa*) which contains reproductive workers, more aggression was shown toward former nestmates with well-developed ovaries than toward immature individuals (Crosland, 1990). The author suggests that this result demonstrates the role of ovarian activity in the development of aggression.

One of the most interesting developments in the assessment of the role of odour cues in ants has been the investigation of the influence of preimaginal learning on subsequent recognition behaviour. In several species of ants it has been determined that the ability to recognize colony odours develops during the larval stage. For example, workers produced from eggs which were transferred to foreign colonies prefer non-sister larvae from their adopted colony over their natural sisters (Isingrini, Lenoir, & Jaisson, 1985); also, carpenter ant workers prefer to lick pupae from the colony that they knew during their larval stage (Carlin & Schwartz, 1989). While it is clear from these studies that ants are

capable of processing information about odours very early in their development, further investigation is required before any conclusions can be drawn as to the source of these odours.

1.3. Olfactory recognition cues in fish and amphibia

The study of individual recognition in vertebrates has focused primarily upon rodents; nonetheless, reports of odour-mediated recognition behaviour in non-mammalian species are becoming more common. This section examines the available literature on fish and amphibia.

1.3.1. Fish

The examination of olfactory cues used in individual recognition in fish has received limited attention. There have been reports of parent-offspring recognition in a number of species (e.g. Myrberg, 1975; Mckaye & Barlow, 1976); however, most interest has focused upon discrimination among siblings. It does not appear that those studies examining the relevance of chemical stimuli as recognition cues have attempted to distinguish between their olfactory and gustatory components. Consequently, the following review illustrates the context within which chemosensory rather than olfactory cues per se have been used to demonstrate individual recognition in fish.

Recognition among siblings has been reported in a variety of tropical fish. For example, juvenile blind cavefish (*Astyanax mexicanus*) choose to move toward the home-aquarium water of familiar rather than non-familiar conspecifics (de Fraipont & Thines, 1986). Sticklebacks (*Gasterosteus aculeatus*) reared in isolation as well as those reared with non-kin only could discriminate between unfamiliar kin and non-kin (Van Harve &

Fitzgerald, 1986). The factors involved in the discrimination process were not assessed in either case.

A number of investigations have assessed the mechanisms of individual recognition in the Salmonidae (Stabell, 1984). Juvenile Coho salmon (*Oncorhynchus kisutch*) discriminated between sibling and non-siblings as demonstrated by their preference for water in the side of a test chamber that had previously been inhabited by siblings. This discrimination was not likely based upon environmental cues as the odours of both unfamiliar and familiar siblings were preferred over unfamiliar non-siblings (Quinn & Busack, 1985). Rather, the authors suggest that discrimination may have resulted from a comparison of the cues produced by conspecifics with a learned phenotypical odour. Further evidence for such a "phenotype matching" hypothesis was provided by the finding that juvenile salmon spent more time in water scented by siblings than water from non-siblings. Salmon that had been raised with both groups, however, showed no difference in the time spent in the water of siblings and non-siblings (Quinn & Hara, 1986). There are several possible interpretations of this result. It may be that the subjects learned the phenotypic odours of different individuals or that the individuals shared a group (school) specific odour. By raising siblings and non-siblings in same and different environments, it might be possible to determine if cues contained in the rearing environment could also contribute to odours of individuality.

Arctic charr (*Salvelinus alpinus*) associated with familiar siblings rather than unfamiliar non-siblings in a Y-maze apparatus and also discriminated between the odours of non-familiar siblings and non-siblings, suggesting the use of genetic based odour recognition cues (Olsen, 1989). The demonstration by Winberg and Olsen (1992) that charr reared in isolation did not discriminate between siblings and non-siblings was taken as evidence that social experience is required to learn recognition cues. The authors suggest

that discrimination is based upon phenotype matching but that self-matching cues provide too restrictive a template and in some instances additional experience with siblings is needed.

Atlantic salmon (*Salmo salar*) were also found to discriminate between siblings and non-siblings but their behaviour was not consistent. Some populations spent more time associating with siblings and other groups avoided their siblings (Stabell, 1982 cited in Blaustein, Bekoff, & Daniels, 1987). An extensive analysis of the type of cues which attracted these salmon to water of conspecifics indicated that the active compounds may be produced in the liver and excreted in fecal material (Stabell, 1987). Neither skin surface extracts nor blood plasma contained discriminable recognition cues. As bile acids from the liver are metabolized by bacteria prior to excretion as volatiles, it is possible that micro-organisms are, in part, responsible for production of the discriminable compounds in the feces.

It may be possible to determine the physiological responses of fish to recognition cues from kin and non-kin. Electro-olfactogram (EOG) recordings have been used to show that fish vary in their olfactory responsiveness to different hormone metabolites (Sorenson, Irvine, Scott, & Stacey, 1992). Recently, molecular cloning of the MHC class I and class II molecules in Atlantic salmon has revealed polymorphic residues similar to those in mice and humans (Grimholt et al., 1993; Hordvik, Grimholt, Fosse, Lie, & Endresen, 1993). Consequently, an examination of the electrophysiological responses of salmon to conspecifics of known MHC types might reveal the role of the MHC in individual recognition in salmon.

The appropriate bioassay required to determine if salmon which differ in MHC type can be discriminated has already been established by Groot, Quinn and Hara (1986). They recorded EOG responses while salmon in a Y-maze chose between water scented with

different excretions from different populations of conspecifics. The magnitude of EOG response was reduced in one population of salmon when water inhabited by individuals from their own population was introduced, indicating that this bioassay is sensitive to differences in individual recognition cues. Thus, the examination of EOG responses of salmon to conspecifics of different MHC types in this paradigm may provide evidence about the relevance of the MHC in kin recognition in salmon.

1.3.2. Amphibia

The study of recognition behaviour in amphibia has generally been limited to an examination of sibling recognition in the larval stage of toads and frogs. Extensive field and laboratory studies of both the Cascades frog (*Rana cascadae*) and the American toad (*Bufo americanus*) indicated that tadpoles associated with siblings rather than non-siblings whether they had been reared with siblings or in isolation (Blaustein & O'Hara, 1981; Waldman, 1981). This ability to discriminate between kin and non-kin when raised in isolation is consistent with the view that recognition of siblings is under genetic control.

Larvae raised in mixed groups of siblings and non-siblings only discriminated between these groups of individuals in a field setting; they distributed randomly among siblings and non-siblings in a laboratory setting (Waldman, 1982; O'Hara & Blaustein, 1985). Waldman (1982) suggested that compared with laboratory experiments, larvae in the field setting may have been exposed to siblings longer during their early development. Thus, familiarity with the odour cues associated with siblings may play a role in the development of the discrimination process.

An extensive investigation of the discrimination behaviour of Cascades tadpoles has revealed that a mechanism other than familiarity may be involved in sibling recognition (Blaustein & O'Hara, 1986). Tadpoles raised with both siblings and non-siblings

discriminated between non-familiar larvae from both groups. Thus, in this instance, familiarity does not appear to be necessary for recognition to occur. Moreover, since tadpoles reared with only non-siblings or in isolation preferred to associate with siblings and Blaustein and O'Hara suggested that experience does not alter the behaviour of tadpoles.

Blaustein & Waldman (1992) have reviewed the evidence for kin recognition in amphibia. They concluded that chemical cues resulting from environmental, genetic and maternal factors are relevant to kin recognition and that kin recognition may facilitate optimal outbreeding. It appears that little attempt has been made to determine the genetic origin of cues of individuality in amphibians. As the levels of polymorphism of the MHC are equivalent to those in rodents (Du Pasquier, Schwager, & Flajnik, 1989), it is possible that MHC-coded signals may be involved in the recognition process.

1.4. Olfactory recognition cues in non-rodent mammals

The existence of characteristic individual odours has been postulated in an extraordinary variety of mammalian species, ranging from bats to dogs to elephants (Gustin & McCracken, 1987; Hepper, 1988a; Rasmussen, Hess, & Haight, 1990). In many instances these reports are limited to one particular context and have not been replicated by other investigators. In contrast, several species including rabbits, deer, tamarins and humans have been studied more extensively. Of special interest are those studies which have attempted to link specific compounds derived from glandular secretions to behavioural responses. Particular attention shall be paid to such literature in the following sections of this chapter.

1.4.1 The rabbit (*Oryctolagus cuniculus*)

In conjunction with the need for pest control, an extensive examination of the composition and function of secretions of the rabbit was undertaken by Mykytowycz and his colleagues in Australia. The systematic analyses of the secretions and glandular homogenates taken from over 1400 rabbits revealed that variations existed in the chemical composition of secretions between individuals (Goodrich & Mykytowycz, 1972). Behavioural observations indicated that the sudoriferous portions of the inguinal glands were important in the recognition of individuals (Mykytowycz & Gooderich, 1974).

In subsequent experiments, the secretions from one animal were rubbed into the fur of another and the frequency and duration of exploratory and agonistic behaviours of the subject and other group members recorded. Hesterman and Mykytowycz (1982a) determined that members of a group of male rabbits smeared with the inguinal gland secretions from male or female rabbits from a different group were attacked by their penmates. Males painted with chin gland secretions and urine from unfamiliar rabbits were not attacked. Female rabbits smeared with the inguinal gland secretions of unfamiliar females but not the secretions of unfamiliar males were also attacked (Hesterman & Mykytowycz, 1982b). The authors hypothesized that inguinal gland odour plays a role in individual recognition as well as sexual identification in the case of female rabbits. Hesterman, Goodrich and Mykytowycz (1981) constructed an airtight animal chamber into which headspace volatiles from fecal pellets were directed. The introduction of volatiles from a male rabbit influenced the outcome of aggressive encounters in pairs of male rabbits in favour of the individual from which the odours were derived. Gas chromatographic fractionation of these volatiles revealed that not all fractions evoked a behavioural reaction in the rabbits (Goodrich, Hesterman, & Mykytowycz, 1981). Among the active compounds were volatile fatty acids similar to those found in the anal glands of the red fox,

Vulpes vulpes (Albone & Fox, 1971) and the coyote, *Canis latran (*Preti, Muetterties, Furman, Kennelly, & Johns, 1976). A behavioural assay should be conducted to determine if these compounds are also involved in the production of odours of individuality in foxes and coyotes.

1.4.2. Ungulates

One of the first and most extensive analyses of the contexts within which specific glandular secretions were correlated with behavioural processes was undertaken by Muller-Schwarze (1971). In an investigation of odour-related behaviour in black-tailed deer (*Odocoileus hemionus columbianus*), he identified pheromonal scents from four glands as well as determining that female urine served as a sex attractant for males. Observations of deer in the wild and in captivity revealed that the tarsal gland of strange deer was frequently investigated by both males and females. Moreover, when the tarsal hairs of a slain deer were rubbed upon the tarsal tuft of a live male or female deer, the subject was approached, followed, sniffed and licked. Muller-Schwarze concluded that scent from the tarsal gland played a role in individual recognition. An application of the distillate to the hind leg of a male deer elicited licking responses, particularly in females. Subsequently, gas liquid chromatography (GLC) analysis coupled with a behavioural analysis revealed that some fractions were more active than others in producing a behavioural response.

The possibility that aldehydes and ketones were responsible for any response was eliminated as precipitating these compounds out of the distillate did not change the behaviour of the deer. Subsequently, it was determined that the most active component of the scent was a lactone compound (Brownlee, Silverstein, Muller-Schwarze, & Singer 1969). It is most interesting, to note, however, that the frequency of responses to fractions of scent increased as the number of components increased (Muller-Schwartze, 1969). This

was interpreted to mean that several substances are responsible for the production of effective odour cues. It also seems that the odours from the other compounds masked the active odour and increased sniffing and licking were required to interpret the recognition signal.

The interest in individual recognition in ungulates has partially arisen from the need to persuade domestic mammals such as sheep and pigs to treat orphaned young as their own offspring (Grau, 1976; Alexander et al., 1986). Operant conditioning experiments have been utilized to determine what glandular secretions or waste products contain discriminable odours. Pigs, goats and calves could be trained to discriminate between the urine odours of conspecifics (Meese, Connor, & Baldwin, 1975; Baldwin, 1977) and sheep could distinguish between urine, faeces, saliva and glandular odours collected from two different individuals (Meese et al., 1975). In a preference test, piglets chose to associate with their mother's urine, faeces or udder odours rather than with the odours of other sows (Horrell & Hodgson, 1992a); moreover, sows could identify their own piglets except when they had been washed with cleaning solution (Horrell & Hodgson, 1992b).

Washing piglets, however, could remove the piglets' own individual odour or the odour of the mother. There have been a number of reports of maternal labelling of young mammals. For example, kid goats which have not been licked or nursed by their mothers will be accepted by any mother, whereas kids which have interacted with their own mother will be rejected by alien mothers (Gubernick, 1980). Mongolian gerbil females (*Meriones unguiculatus*) will accept strange pups smeared with the female's own ventral gland secretions (Wallace, Owen, & Theissen, 1973) and female rabbits will attack their own young if their fur is rubbed with inguinal gland secretions from an alien female (Mykytowcyz, 1968). Such findings suggest that mother-infant recognition may be a result of mothers marking their young with their own secretions and recognizing these cues

rather than those of their offspring. If cross-fostering techniques can be successfully initiated, it should be possible to test this hypothesis.

1.4.3. Carnivores

Both behavioural and chemical analyses have been used to demonstrate the presence of individually distinct odour cues in carnivores. For example, a habituation-discrimination technique was used to show that dogs (*Canis familiaris*) and wolves (*Canis lupus*) could discriminate between the urine odours of conspecifics (Brown & Johnston, 1983); a gas chromatographic (GC) headspace analysis followed by a mass spectrographic (MS) identification of the anal sac secretion of three species of hyaena, *Hyaena brunnea*, *H. hyaena* and *Crocuta crocuta* indicated that there are both quantitative and qualitative differences among the anal gland secretions of these species (Buglass, Darling, & Waterhouse, 1990). Neither of these studies combined the chemical and behavioural analyses as has been done in the investigation of scent-marking and odour discrimination in the mongoose.

Extensive observations of scent marking in the African Dwarf mongoose (*Helogale undulata rufula*) led to the hypothesis that anal gland but not chin gland secretions provided individual recognition cues (Rasa, 1973). The results of a choice experiment in which a mongoose was trained to sniff one secretion and then to choose a "match" from a series of scents confirmed that the animals could distinguish between the anal gland but not the chin gland secretions of individual conspecifics. Following this study, a chemical analysis of the anal gland secretions of a different species, the Indian mongoose (*Herpestes auropunctatus*) revealed that carboxylic acids differed both in overall and relative concentration in different individuals (Gorman, Nedwell, & Smith, 1974).

Gorman (1976) trained tame Indian mongooses to discriminate the odours of anal glands of individual conspecifics in a matching-to-sample design. The animals were presented with one glass slide smeared with an odour to sniff. They received food reinforcement for choosing an odour identical to the original odour from among a number of odorized slides. They could discriminate between the anal gland secretions of different individuals as well as between synthetic mixtures of these acids.

Although these studies suggest that the anal gland secretion is involved in discrimination of individuals, the source of the chemical difference cannot be conclusively attributed to the identified carboxylic acids. The Egyptian mongoose (*Herpestes ichneumon*) also showed individual and sex differences in anal gland secretions. A GC-MS analysis revealed qualitative differences in long-chain carboxylic acids rather than in the saturated carboxylic acids found in the Indian mongoose (Hefetz, Ben-Yaacov, &Yom-Tov, 1984). Regrettably, no bioassay was included in this study.

Analyses of scents from other mammals have identified a number of constituents which may provide odour recognition cues in carnivores. For example, sulfur containing compounds have been found in the anal sac secretions of mustelids including mink, otter, stoats and weasels (Brinck, Erlinge, & Sandell, 1983). The anal sacs of the red fox (*Vulpes vulpes*) contain fatty acids and also amines (Albone & Perry, 1975). Clearly, without a behavioural assessment, no hypotheses about the function of these volatiles can be developed. While the majority of studies have focused upon the role of anal gland secretions, it is likely that chemicals from other glands also participate in the formation of an individual odour profile. Considering that both ants and humans excrete some of the same products, e.g. 4-heptatone, 4-methyl-2-pentanone, (Ellin et al., 1974; Hefetz & Lloyd, 1983), it m y be more appropriate to focus upon the odour profile and its origins rather than the individual components of odours.

1.4.4. Non-human Primates

Non-human primates use olfactory cues to communicate information regarding their sexual and social status. For example, the odour of vaginal secretions is responsible for female attractiveness in the rhesus macaque (*Macaca mulatta*) (Keverne, 1983) and urine washing is associated with dominance interactions and reproductive condition in squirrel monkeys (*Samiri scirus*)(Epple, 1985). There have also been reports of individual recognition based upon characteristic odours in flying phalangers (*Petarus breviceps papuanus*) (Schultze-Westrum, 1969); the greater bushbaby, *(Galago crasicaudatus*) (Clark, 1978); the squirrel monkey (Kaplan, Cubicciotti, & Redican, 1976); the ring-tailed lemur (*Lemur catta*) (Mertl, 1975) and the woolly spider monkey (*Bracyteles arachnoides*) (Milton, 1985). The sources of the odour vary and include wrist, frontal and sternal glands and urine.

Among the most extensively studied of the primates is the saddle-back tamarin (*Saguinus fuscicollis*). Epple and her colleagues have used both behavioural tests and chemical analyses to investigate the role of scent marks in individual recognition (See Epple & Smith, 1985 for a review). They also developed a computerized pattern recognition technique which provided a precise method for evaluating the similarities in samples obtained from high resolution gas chromatographs (Smith, Belcher, Epple, Jurs, & Lavine, 1985).

Initially, in a series of preference tests, Epple (1979) showed that scent marks can be used to discriminate between individual tamarins. A GC-MS analysis of the supracaudal gland secretions found in scent marks revealed individual differences in the concentration of squalene, a lipid compound, and in the amounts of butyrate esters present in marks from different individuals (Yarger, Smith, Pretti, & Epple, 1977). Since individual scent marks showed a consistent squalene/butyrate ratio when the scent marks were sampled daily for

seven days, the authors suggested that an "ester profile" may provide the information necessary to create an individual identity (Epple, Golob & Smith, 1979).

Epple and Smith (1985) reported further evidence of consistent profiles in the scent marks of several animals collected over a one year period. In behavioural choice tests, using synthetic ester preparations, however, the tamarins examined the samples at random, an indication that the squalene/butyrate mixture was not sufficient for individual recognition. Belcher et al. (1986) found that the squalene/butyrate ester fractions could not be discriminated without the inclusion of an unidentified highly volatile glandular component.

Such findings emphasize the need for an assessment of the behavioural significance of specific glandular secretions prior to attributing any importance to their role in individual recognition. Nonetheless, the careful evaluation of the relationship between scent marking and individual recognition in tamarins by Epple and her colleagues has provided a model for investigation of odour-mediated behaviour in all species.

1.4.5. Humans

Visual cues are most relevant for identification of individual humans. Although there is not an extensive experimental literature to justify this statement, the neuropsychological reports do support the hypothesis that specific visual mechanisms are devoted to recognizing faces. Individuals who have bilateral damage to specific areas of their medial occipito-temporal regions suffer from and can no longer identify familiar individuals or learn to recognize unfamiliar face. The ability of these people to recognize objects is not impaired (Etcoff, Freeman, & Cave, 1991). Moreover, cells in the temporal cortex of monkeys have been found to respond selectively to faces (Desimone, 1991). Thus, primates are unlikely to require olfactory cues to identify individuals. Despite, or perhaps

because of, our limited need for olfactory communication, however, the study of odour-mediated cues in humans has received a considerable amount of attention. The discriminability of odours from unrelated individuals as well as those from kin have been assessed.

Individuals discriminated between the T-shirt odours of males and females (Russell, 1976; Hold & Schleidt, 1977; Wallace, 1977). When choices increased from two to ten, it became more difficult to make the male-female discrimination (Hold & Schleidt, 1977). Errors were generally the result of classifying strong or pungent odours as male (Doty, Orndorff, Leyden, & Kligman, 1978; Hold & Schleidt, 1982).

Unknown individuals of the same sex were discriminated by their hand odours but it was, however, difficult to discriminate between the odours of monozygotic twins (Wallace, 1977). As the odours of identical twins eating different foods were easier to discriminate, it appears that dietary cues can also facilitate odour discrimination in humans. In one other report, women without children were able to pick out the undershirt of an infant they had held for 45 minutes even though they were unaware that they would be tested after the interaction (Kaitz & Eidelman, 1992). Wallace (1977) appears to have provided the only report of humans discriminating same-sex individuals with whom they have had no prior experience.

Odour recognition among humans has been extensively examined by Porter and his colleagues. They determined that, in a two choice task, both mothers and fathers could discriminate between the odours of T-shirts worn by their own children (Porter & Moore, 1981) and strangers could match the odours of mothers and their children but not the odours of husbands and wives (Porter, Cernoch, & Balogh, 1985). If the matches had been made as a result of environmental similarities, matches would also have been made in

the case of husbands and wives. Thus, these results support the hypothesis that the individual odours of humans are genetically based.

Grandmothers and aunts could also successfully discriminate between the clothing odours of their newborn relatives and unrelated strangers (Porter, Balogh, Cernoch, & Franci, 1986). These subjects had limited exposure to the infants but were also told of the purpose of the experiment several hours prior to the test. As Porter et al. (1986) point out, the subjects would have had the opportunity to familiarize themselves with the odours of the infant, during this time. Testing the relatives prior to their interacting with the infants would provide more substantive evidence of the genetic hypothesis of odour recognition in humans.

Another series of experiments focused upon the ability of infants to discriminate between the odours of their own mother versus those from an unknown lactating female. Macfarlane (1975) and Russell (1976) found that infants oriented toward their own mother's breast odour but not toward the breast odour of a strange mother. Also, infants reduced their body movements more in the presence of their own mother's breast odour compared with the odour of an unfamiliar mother (Schaal et al., 1980, cited in Schaal & Porter, 1991). Whether infants use cues learned in utero or postnatally cannot be determined from the latter experiments.

Breast-fed babies also discriminated between the axillary odours of their mother and those of an unfamiliar lactating female whereas bottle-fed babies could not discriminate between the odours of their own mother and another unfamiliar bottle-feeding female (Cernoch & Porter, 1985). This would suggest that the breast odours are learned during breast-feeding. When given a choice between the breast odours of a lactating and non-lactating woman, bottle-fed infants oriented toward the breast odour of the lactating mother

(Makin & Porter, 1989). Thus, it would appear that breast odours of lactating mothers have a general attraction for neonates.

The ability of mothers and fathers to identify their own newborns has also been assessed. Mothers can identify the odour of their own infant or their shirts after only a 30 minute exposure to their own infant (Russell, Mendelson, & Peeke, 1983; Porter, Cernoch, & Mclaughin, 1983; Kaitz, Good, Rokem, & Eidelman, 1987). On the other hand, the evidence for father-infant recognition is equivocal. In one study, fathers were not able to discriminate between the head odours of their own and two other infants (Russell, Mendelson, & Peeke, 1983); whereas in another study in which fathers had to choose between the undershirt odours of two infants, they correctly identified their own child (Porter, Balogh, Cernoch, & Franci, 1986).

Kaitz et al., (1987) claimed that they had eliminated the confounding of mothers' and infants' odors by testing the mothers with a garment that had been worn under a hospital gown. It is certainly true that they kept the postnatal exposure to a minimum; however, it is impossible to eliminate the influence of the mothers' prenatal environment upon the infant. With the current trends in reproductive technology, it may be possible to separate genetic influences from those of the mother by testing the ability of surrogate mothers to identify the infants they have carried.

Testing human subjects could provide the opportunity to obtain anecdotal evidence about the perceived nature or source of odours of individuality; however, the literature does not contain such information other than a report that mothers indicated that their infants breath was the source of their individual odour (Russell, Mendelson, & Peeke, 1983). Volatile products are also emitted from saliva, sweat, sebum, dermal cells, urine, feces and flatus (Ellin et al., 1974) but it is most likely that only oral (breath and saliva) and skin (sweat and sebum) odours are generally available for sampling. Based upon an

extensive analysis of surface lipids of the skin, it was hypothesized that individuals were unique with regard to the proportions of fatty acids that their skin produces (Nicolaides, 1974).

While it seems likely that human odours are influenced by a combination of genetic and environmental factors, such an hypothesis has not been investigated. Moreover, humans have a very limited capacity to identify individual odours in mixtures, i.e., it is very difficult for them to identify more than three individual components of a mixture (Laing & Francis, 1989). Consequently, it is possible that a person's discernable odours blend into a composite odour which is perceived as characteristic of that individual. To gain a better understanding of the nature of human olfactory cues, it would be appropriate to conduct interviews with experimental subjects. In particular, it may be valuable to assess prosopagnosiacs who may make greater use of olfactory recognition cues to identify both familiar and non-familiar individuals.

The manner in which human odours are perceived has been extensively investigated using dogs as the discriminators. In fact, the idea that "it would be an interesting experiment for twins who are closely alike to try how far dogs could distinguish them by scent" was proposed by Galton in 1875 (cited in Kalmus, 1955). Eighty years later, the experiment was conducted (Kalmus, 1955). In retrieving experiments, a trained dog was able to correctly choose a handkerchief scented with the axillary odour of an individual who had previously clasped the dog's muzzle. If the odour of the identical twin was included among the choices, the dog retrieved either of the two handkerchiefs at random. In tracking experiments, however, the dog could discriminate between the scents made by identical twins. It was suggested that the difference in behaviour could be accounted for by the method of presentation of the odours in the tracking experiment. When confronted

with the odours from identical twins simultaneously, the dog could make a more subtle distinction based upon environmental factors such as diet, personal hygiene or clothing.

The various factors associated with human odour cues were examined by Hepper (1988a). In a matching-to-sample simultaneous discrimination task, dogs could discriminate between the T-shirt odours of dizygotic twins on the same diet but not between the odours of monozygotic twins on the same diet. The odours of adult identical twins who lived apart and consumed different foods could be discriminated. As the dogs could not discriminate between T-shirts which had been left in the subject's homes, it appears that odours from external sources did not contribute to the individual's odour profile. While this study provides convincing evidence that both genetic and dietary cues contribute to individual odour identification, it does not address the source of these odours. It is possible that differences in both genetic and diet factors result in quantitative or qualitative differences in bacteria which could in turn influence odour production.

1.5. Summary

The experiments reviewed in this chapter have provided evidence that olfactory cues may be used to discriminate between individuals in both invertebrate and vertebrate species. In some instances, the cues appear to be mainly of genetic origin and in other cases, to be derived from the environment. In those cases where the components of the odours were analyzed, the end products generally included volatile short chain fatty acids, aldehydes and ketones. This latter finding was consistent regardless of the species studied or the odour source examined. Thus, it would appear that, individual odour profiles may, in part, differ quantitatively rather than qualitatively from the profiles of others.

CHAPTER 2

EMPIRICAL EVIDENCE FOR ODOURS OF INDIVIDUALITY
PART 2: THE RODENTS

The search for olfactory cues of individual recognition owes much of its impetus to the experimental analysis of behaviour in rodents. The literature on rodents illustrates the theoretical issues involved, the methods used, and the results obtained in assessing the ability of animals to use odour-based cues to discriminate between individuals. Consequently, this chapter is devoted to a comprehensive examination of individual recognition as exemplified by olfactory signals produced by rodents. Particular emphasis is placed upon the different contexts in which discriminable cues have been demonstrated and the factors which may influence the outcome of the discrimination process.

2.1. Factors contributing to individual odour profiles

It would be unrealistic to hypothesize that the odour profile of an individual remains constant throughout its lifetime. One might propose that individual odours consist of both invariant components and a number of changing cues both of which could vary in intensity over time. Since we do not know whether an animal perceives an individual's odour as a whole in a Gestalt-like fashion or as a set of different cues (an odour pattern), it is difficult to know how an animal adapts to changes in odour components. The results of one recent study have shown that, for fruit-eating bats, the threshold for detection of an odour mixture is much lower than the threshold for its individual components (Laska, Hudson, & Distel,

1990). The enhanced detection of the compound suggests that, in some instances, an odour mixture produces its own unique cue.

Another possibility is that the constant cues provide a background which enables the presence or absence of variable cues to be detected without any loss of awareness of who the individual is, analogous to the way in which the visual image of an individual provides both constant and variable cues (e.g. eye colour versus hair length). Alternatively, Halpin (1986) has hypothesized that non-odour cues may provide sufficient recognition cues for an animal while changes in odours are being learned and associated with an individual. Regardless of the processes involved in the "assembly" of the odour, the experimental literature provides evidence that, in rodents, odour cues of both a constant and variable nature are discriminable.

Odours which are the product of constant genetic differences appear to be used by animals to recognize their kin (Hepper, 1987a) and are also involved in mate choice (Boyse, Beauchamp, Bard, & Yamazaki, 1991). In addition, odours which are a result of genetic differences can be used by non-kin to discriminate between two related or unrelated individuals (Schellinck, Brown, & Slotnick, 1991). In this chapter, the availability of odour cues for use in kin discrimination will be described in the contexts of parent-offspring, offspring-parent odour recognition and the discrimination of siblings. The ability of non-kin to discriminate between the genetically-derived odours of conspecifics will also be assessed. Mate choice studies which have assessed the discriminability of odours in which the degree of relatedness of the odour donors to the odour discriminators is known (e.g. siblings, 1st cousins, 2nd cousins) will also be reviewed. The role of the MHC in mate choice will be reviewed in Chapter 4.

Variable odours which are present in some instances and absent in others have been demonstrated to generate discriminable cues in several contexts. For example, in many

rodent species, males use odour cues to discriminate between estrous and non-estrous females (Brown, 1979; Johnston, 1983a). Furthermore, variability in the degree of concentration or intensity of any one odour may also be detected. In one study, the discriminability of the odours of dominant and subordinate males was shown to be based upon comparison of levels of intensity of testosterone-mediated odours (Taylor, Haller, & Regan, 1982). Odours attributed to changes in diet have also been found to provide discriminable odour cues (Beauchamp, 1976). In this chapter, experiments which have evaluated the discriminability of estrous odours in females and odours of dominance in males will be described. Investigations which have assessed the relevance of diet cues to odour discrimination will be considered in Chapter 5.

2.2. Discrimination of female estrous states by males

The ability to assess the reproductive condition of a potential mate is considered a crucial aspect of sexual behaviour (Johnston, 1980) and the ability to do so has been examined extensively in laboratory studies. When trained in a Y-maze for water reinforcement, both intact and castrated male rats (*Rattus norvegicus*) which were sexually naive could discriminate between the odours of estrous and non-estrous females (Carr & Caul, 1962). In a preference test, sexually experienced males, but not sexually naive or castrated males, showed a preference for the odours of estrous females (Carr, Loeb, & Dissinger, 1965). Thus, although both sexually naive and castrated rats can be trained to discriminate between odours of estrous and non-estrous females, this ability may not reflect their behaviour in a natural situation.

For the most part, the results showing that male rats prefer the odour of estrous to non-estrous females have been replicated (Pfaff & Pfaffman, 1969; Stern, 1970; Landauer,

Wiese & Carr, 1977). Nonetheless, in a comprehensive investigation of odour preferences, Brown (1977) found that neither sexually naive nor experienced male rats showed a preference for the urine of estrous over diestrous females but that sexually experienced males were more attracted to the odours of all females than sexually naive males. He pointed out that test duration, amount of the test stimulus and type of previous sexual experience are all factors to be considered in any comparative analysis of preference test results.

The source of the odour differences between estrous and non-estrous female rats has been attributed to both the preputial gland secretions and the urine. Gawienowski, Orsulak, Stacewicz-Sapuntzakis and Pratt (1976) examined the preferences of male rats for volatiles extracted from the preputial glands of the female rat and determined that sexually experienced males preferred the preputial odours of intact or estrogen and progesterone primed ovariectomized females over the odours of ovariectomized females. Such findings confirm the role of sex hormones in the production of the odour of estrous females.

Female urine is also a source of odours indicating estrous state. Perhaps indicative of its relevance in revealing reproductive state comes from the report that male rats can detect one part estrous urine in 100,000 parts water (Carr, Solbey, & Pfaffman, 1962). Sexually experienced but not inexperienced rats preferred urine odours from estrous females to those from diestrous females (Lydall & Doty, 1972). The male rats could discriminate estrous state whether bladder urine or normally voided urine was used. As bladder urine is free of microflora, this finding suggests that external bacteria are not required for the production of discriminable differences in urine odour. However, it should be noted that this finding does not in any way dismiss the role of gut bacteria in the production of metabolites which may provide discriminable cues in urine.

A comparison of the results of the rat studies with those from other rodents reveals that odour preferences are not consistent across or within species and may depend upon the experimental manipulations involved. Sexually experienced male mice (*Mus musculus*) showed a preference for whole body odours of estrous over diestrous females but sexually naive males had no demonstrable preference (Hayashi and Kimura, 1974). These results were not replicated by Rose & Drickamer (1975) who found that both sexually experienced and sexually naive mice showed a preference for the odours of estrous over non-estrous female mice. Urine odours rather than odours emanating from the whole animal were used in the latter experiment; also, the mice were from different strains and were tested at different ages. Thus, it is possible that different methodologies led to the conflicting results.

Male golden hamsters (*Mesocricetus auratus*) show a preference for the whole body odours of estrous versus diestrous females only if they are sexually experienced and only when the donor animals are not present (Landauer, Banks & Carter 1978; Johnston, 1983a). While this result may seem unusual, Huck, Lisk, Kim and Evans (1989) presented evidence to suggest that dilution of whole body odours in air may enable the discrimination of odour differences that would otherwise be undetected because of the large volume of odours emitted at close range. In a four-choice test, male hamsters showed no preferences among undiluted female odours but had significant preferences in the order diestrous 2 > proestrous > estrous > diestrous 1 when the odours emanating from the females passed through an air dilution apparatus prior to reaching the test animals.

These conflicting results could be interpreted in terms of Nyby's (1983) hypothesis that chemosignals of low volatility detected by the vomeronasal organ may affect male behaviour when the male is close to the female whereas volatile signals detected by the olfactory bulb are linked to preference at a distance.

In a comprehensive study, male deer mice (*Peromyscus maniculatus*) showed no preference for the odours of estrous versus diestrous females (Dewsbury, Ferguson, Hodges & Taylor, 1986). The authors suggested that the presence of a female may be more important than her degree of sexual receptivity. As these animals are seasonal breeders, their laboratory performance may not be indicative of their true preference unless they were tested during the appropriate breeding season or in the appropriate photoperiod.

In a number of other species, including lemmings (*Dicrostonyx*), Columbian ground squirrels (*Spermophilus columbianus*), gerbils (*Meriones unguiculatus*) and desert wood rats (*Neotoma lepida*), males prefer the odours of estrous to diestrous females (Huck & Banks, 1984; Dewsbury et al.,1986). In general, the role of experience appears to play the deciding factor in the choice of the males with sexually experienced males showing a preference for estrous females whereas sexually naive males do not.

2.3. Discrimination of dominance status

The concept of dominance is central to understanding social behaviour in many species of mammals. Depending upon the definition that one adopts, the discrimination of individuals may or may not be considered to be a part of the dominance relationship. If one characterizes dominance as an asymmetry in the outcome of diverse agonistic interactions resulting in avoidance of escalated encounters and postulates that past encounters influence subsequent responses then, individual recognition is a necessary component of the process (Drews, 1993). In the following section, the evidence that odours of dominance are discriminated by both males and females will be presented. The results of studies which have investigated the source of these odours will also be reviewed.

2.3.1. Discrimination of dominant and subordinant males by other males

Krames, Carr and Bergman (1969) established that an odour was associated with dominance status by demonstrating that male rats living in a group with a known dominance hierarchy spent significantly more time investigating the odours of submissive males rather than dominant males. While rats in unstable groups also investigated the odours of submissive males longer, the difference was not significant. The authors hypothesized that subjects found the odours of dominant males to be aversive.

Scent-marking activities are also indicative of the ability of subordinate males to discriminate between the odours of other individuals. Subordinate rats scent-marked less near the odour of the dominant male after losing an aggressive interaction with such an individual (Adams, 1976). Similarly, gerbils which lost aggressive encounters with a colony resident scent-marked less in the presence of the resident's odour (Nyby, Thiessen, & Wallace, 1970).

Several studies which have demonstrated the discriminability of odours from dominant and subordinate individuals have not taken into account that the odours from one of the individuals were more familiar to the subject (Nyby et al., 1970; Carr et al., 1970; Poole & Morgan, 1975). Thus, it is not possible to say whether the discrimination was based upon recognition of a familiar odour or the odour of dominance. In an attempt to eliminate degree of familiarity as a confound in the discrimination of the odours of dominant and subordinate individuals, South American cavies (*Cavia aperea*) were tested with odours from different members of their own colony with whom their dominance relationship was already known (Martin & Beauchamp, 1982). The test animals spent more time on the side of a cage containing the odours of the subordinate male. The authors also reported unpublished work which indicated that male cavies did not respond differentially to the odours of unfamiliar dominant and subordinate individuals. Martin and

Beauchamp suggested that their results provide evidence that the test animals are responding to cues of individual recognition rather than to categories of dominance.

All of the studies so far described have examined the responses of rodents to the dominant and subordinate odours resulting from dyadic interactions. In a study of dominance in domestic guinea pigs (*Cavia procellus*), Drickamer and Martan (1992) assessed the rank of guinea pigs in groups of three or four prior to examining the response of each male to swabs from the perineal region of other males in the group. They were able to determine that there was a significant linear relationship between dominance rank and the proportion of aggressive responses. To confirm that the guinea pigs used cues of individual recognition rather than only discriminating relative dominance, it would be necessary to assess their response to the odours of unknown individuals in established groups.

Despite Martin's and Beauchamp's (1982) claim that familiarity is a confound in assessing individual recognition cues, Brown (1992) argues that the very fact that dominant resident male rats spent more time urine marking and investigating the odours of familiar subordinate intruders but not unfamiliar subordinates means that they recognize this odour when it is encountered in a neutral arena. He suggests that because one subordinate odour is treated differently than another, the rats are not using the categories of dominance for discrimination. While this may be true, others would clearly argue that the rats are using the categories of familiar and unfamiliar to make the discrimination. This seems to beg the question of what recognition really is: If one adopts the stance that odours of individuals are learned so that the individuals can later be recognized, then it seems that familiarity should not be considered a confound but the causal factor in this equation.

2.3.2. Discrimination of dominant and subordinant males by females

The ability of females to discriminate between male odours based upon the social status of the individuals may influence their selection of a potential mate. The choice of a dominant male as a mate could result in fitness benefits for the female (Dewsbury, 1982a; Bateson, 1983). From, this perspective, it would not be necessary for females to recognize prospective mates as individuals; rather they could merely be categorized as dominant or subordinate. Nonetheless, since males will always bear an odour characteristic of their status, it seems likely that this odour would be incorporated into their unique odour identity.

A variety of paradigms have been used to determine if female rodents are able to discriminate between the odours produced by males as a result of their dominant-subordinate relationship. For example, in a Y-maze olfactometer, estrous female hamsters spent more time near the urine odour of a dominant male (White, Fischer & Meunier, 1984a) as well as preferring the flank gland odours of dominant males to those of subordinate males (Montgomery-St. Laurent, Fullenkamp, & Fischer, 1988). Estrous female lemmings (*Lemmus trimucronatus*) choose to stay in the arm of a Y-maze containing the whole body odours of a dominant rather than a subordinate male (Huck, Banks, & Wang, 1981).

In an open field test, estrous female mice spent more time investigating the urine of dominant mice, when given a choice between clean paper and urine-scented paper; the duration of investigation of clean paper and the urine of subordinate males did not differ significantly (Jones & Nowell, 1974). Similarly, female mice were attracted to the actual bedding as well as the pumped in bedding odours of dominant rather than subordinate males (Drickamer, 1992).

When given a choice between two teathered males in a T-maze, female rats spent more time with and mated more frequently with the dominant male of a dyad (Carr, Kimmel, Anthony, & Schlocker, 1982). The role of odours in the discrimination process was not assessed. As female rats can discriminate between the whole body odours of normal and castrated males (Carr, Loeb, & Dissinger, 1965) and the bedding odours of individual males (Krames, 1970), it seems that odours may have been sufficient to produce the results in Carr et al.'s (1982) study.

When assessing the response of females, it is important to realize that their attraction to the odours of males may change depending upon their hormonal state. Diestrous female hamsters spent more time investigating the urine odours of subordinate males than those of dominnant males (White et al., 1984). Similarly, diestrous lemmings stayed significantly longer in the side of a Y-maze containing the whole body odours of subordinate males (Huck, Banks & Wang, 1981).

Whether the females were attracted to the subordinate's odour or were avoiding the dominant's odour could not be determined from either of these experiments. The urine of dominant mice is aversive to subordinate males as well as to other dominant males (Jones & Nowell, 1974); thus, it is possible that a similar response is produced in non-receptive female hamsters by the urine of dominant males. Regardless of the cause, such results serve to emphasize the potential influence of the hormonal state of females upon their attraction to odours.

2.3.3. What are the sources of odours of dominance?

Odours associated with social status have frequently been found in urine. They are also discriminable in saliva and the secretions of ventral, flank and perianal glands of some species of rodents (Freidle & Fischer, 1984; Montgomery-St. Laurent, Fullenkamp, &

Fischer, 1988; Drickamer, 1992). Evidence from experiments which have specifically manipulated testosterone levels of the odour donors indicate that odours of dominance appear to be based on individual differences in odours mediated by changes in testosterone. For example, when given a choice between the odours from two intact males, female rats preferentially marked the odour of the male with the highest level of testosterone (Taylor, Haller, & Regan, 1982).

Nonetheless, as the high levels of stress experienced by subordinate males dramatically influences both their endocrine and immune systems (Brown, 1994), it would be simplistic to hypothesize that the odour discrimination is based upon differences in testosterone metabolites alone. Consequently, there could be quantitative and qualitative changes in a number of different odorants. An increase in adrenocorticotrophic hormone (ACTH) from the anterior pituitary will result in changes in ACTH metabolites in the urine as well as those of testosterone. As ACTH has a major influence upon the production of glucocorticoids in the adrenal cortex, their metabolites will also be excreted in the urine. Changes in corticosteriod levels have been demonstrated to influence the odours of humans (Kloek, 1961) and may be discriminable in rodents as well.

Because of the increased activity of these steroids, the functions of the immune system will be inhibited. Subordinant males are, therefore, more susceptible to disease and, as a result, may be infected with odour-producing organisms. There has been both anecdotal and experimental evidence that changes in bacterial flora and parasite infestations associated with disease may produce changes in body odour (Bedichek, 1960; Liddell, 1976; Kavaliers & Colwell, 1993). Thus, it seems that odours of dominance may be a result of complex interactions in the neuroimmunoendocrine system.

There is some indication that the odours related to dominance may have a genetic basis. If dominance is defined as a relationship between two individuals, it is clear that

dominance per se cannot be inherited; however, Dewsbury (1990) has suggested that a capacity to become dominant may be passed from parent to offspring. If dominance has a genetic basis, it may be associated with an unchanging odour component. Several studies have provided support for this position. Both estrous female lemmings and hamsters spent more time investigating the odours of socially naive males which subsequently became dominant in encounters with other males (Huck, Banks, & Wang, 1981; White, Fischer & Meunier, 1984b). Moreover, female mice spent more time investigating the soiled bedding odours from the sons of dominant males which had not had any social experience over the odours from sons of subordinate males. (Drickamer, 1992).

Heightened aggressive behaviour is generally associated with dominance and mice can be selectively bred for degrees of aggressiveness (Sandnabba, 1985, 1986a, & 1986b). The response of male and female mice to the odours of aggressive and non-aggressive males is similar to their response to the odours from dominant and subordinate individuals. For example, bystander males avoided the odours of aggressive males; females investigated these same odours (Sandnabba, 1985; 1986a). In addition, urine from aggressive males provoked an aggressive response when applied to castrates whereas their bedding suppressed aggressiveness (Sandnabba, 1986b). An increase in urinary marking also correlated positively with degree of aggressiveness (Sandnabba, 1985); a similar correlation was also found between urine marking and dominance status (Ralls, 1971; Adams, 1976; Hebert & Barette, 1989; Brown, 1992). Thus, it seems likely that aggressiveness could be the inherited characteristic associated with dominance.

Specific testosterone dependant volatiles which may contribute to the odours of dominant mice have been identified in urine by GC-MS (Novotny, Schwende, Wiesler, Jorgenson, & Carmack, 1984; Schwende, Wiesler, Jorgenson, Carmack, & Novotny, 1986). More than 61 volatiles were identified in the urine of mice (Schwende et al., 1986)

and 16 of these showed significantly different concentrations between dominant and subordinate individuals (Harvey, Jemiolo, & Novotny, 1989). When added to the urine of castrated males, two compounds, 2-sec-butyl-dihydrothiazole and dehydro-exo-brevicomin caused an increase in the amount of time that females spend investigating the urine odours (Jemiolo, Alberts, Sochinski-Wiggins, Harvey, & Novotny, 1985).

A comparison of the gas chromatographic profile of the whole body odours of mice with the number of aggressive encounters initiated has indicated that the odour profile agrees with aggression initiator's rank but not always with encounter winner ranks (Apps, Rasa, & Viljoen, 1988). The authors suggest these results may indicate that their subjects were not yet in stable dominance relationships. What is particularly interesting about the GC analysis is the demonstration that the whole body odours of individual mice differed both quantitatively and qualitatively in a number of identified peaks, thus indicating the presence of an odour profile of individuality.

2.4. Discrimination of kin

The idea that animals may have the ability to recognize kin has generated much debate and appears to have given rise to more theoretical literature than any other single topic in animal behaviour (e.g. Kurkland, 1980; Holmes & Sherman, 1983; Fletcher & Michener, 1987; Hepper, 1991a). The review by Waldman (1988) provides a particularly thorough analysis of the contexts and mechanisms of kin recognition. In some instances, the question under consideration is not only the nature of the evidence which supports this concept but also whether or not kin recognition actually exists (Grafen, 1990; Byers & Bekoff, 1991). Such a debate commands theoretical interest because of the issues raised in

Hamilton's (1964) theory of inclusive fitness and Bateson's (1983) model of optimal outbreeding.

Hamilton's model predicts that close relatives will be the recipients of more altruistic acts than distant relatives, thereby promoting the individual's inclusive fitness. Bateson (1983), by analyzing the costs and benefits of inbreeding and outbreeding, determined that the optimal mate to choose is a relative who is somewhat different but not too different from one's self. The ability to discriminate between kin and non-kin or kin of different degrees of relatedness is required for both of these concepts to be operationalized. Thus, studies which reveal the ability of an animal to discriminate between the odours of related individuals have the potential to provide support for both theories.

Experimental evidence for kin recognition is not particularly common, however, and there are a number of difficulties associated with such research. For example, as a result of differing operational definitions, investigators may disagree as to when kin recognition has been demonstrated. Grafen (1990) indicates that "species recognition and group member recognition systems may produce an ability to discriminate conspecifics by genetic similarity on first encounter when kin recognition is absent" (p. 42). He suggests that an individual is likely to use itself or those surrounding it as exemplars of species and it will therefore choose kin as representative of species. Thus, the kin recognition process is only a side effect of the individual/family recognition systems and should not be considered otherwise. Grafen (1990) states that kin recognition requires not only polymorphism at matching loci but also "individuals to use sensory information to make decisions about social behaviour in which the role of sensory information is to ensure an accurate assessment of relatedness" (p. 53).

According to Blaustein, Bekoff, Byers and Daniels (1991), Grafen's (1990) definition, makes it is impossible to discriminate between kin and species recognition.

They do concede, however, that other than in the case of alarm calls in squirrels (Schwagmeyer, 1980; Holmes & Sherman, 1982, Holmes, 1984a), there is no evidence for any adaptive value of kin recognition in any of the commonly cited reports of "kin recognition" (reviewed in Section 2.2.2.).

Byers and Bekoff (1991) argue that Grafen's (1990) conceptualization is simplistic as he does not consider the role of behavioural development in the kin recognition process. They suggest that phenotypic variance may be a composite of environmental as well as genetic components and that these factors may influence differential treatment of conspecifics and could also lead to kin recognition. The consideration of environmental influences could also account for individual recognition among such kin as sibling littermates.

Even those investigators who are confident that kin recognition is a general phenomenon are not always in agreement as to the process by which it is expressed. The four different mechanisms which have been proposed to explain how kin are recognized have been summarized by many authors (Fletcher & Michener, 1987; Hepper, 1991b). They are as follows: 1. Spatial location: Kin are recognized because of the environmental cues associated with them such that a conspecific within a specific physical area is considered kin.

2. Association or familiarity: Individuals learn to recognize the cues of conspecifics such as parents or siblings whom they commonly encounter and respond to them as kin. Hepper (1991b) suggests that this form of recognition relies on the use of cues of individuality.

3. Phenotype matching or the "armpit effect" (Dawkins, 1982): Individuals form a recognition template based upon learning their own cue or that of relatives and match the cues of encountered individuals to the template. The degree of matching will determine the

coefficient of relatedness. The difference between phenotype matching and recognition by familiarity is that in the former an individual can "recognize" a conspecific that it has not met. As Hepper (1991b) points out, however, association or familiarity may be part of the process by which phenotype matching occurs, thus, it may not be appropriate to consider them as two different mechanisms.

4. Recognition genes: An individual is genetically predisposed to recognize kin and requires no experience with a conspecific to do so. Crozier (1987) has distinguished between two types of recognition genes: "green-beard" alleles and recognition alleles. "Green-beard" alleles (Dawkins, 1982) refer to a gene which simultaneously codes for a discriminable phenotype, the ability to recognize the phenotype and an altruistic response toward the bearer of the phenotype (Hamilton, 1964). Recognition alleles determine a characteristic cue and the ability to recognize it but do not code for any behavioural response. Phenotype matching is different from the latter concept in requiring that an individual must learn the matching cues rather than having the innate ability to do so. If phenotype matching uses self as a referent, however, there is no way to discriminate between the two concepts.

Recently, definitions which acknowledge the contribution of learning as well as the importance of both genetic and environmental cues in kin recognition have been proposed. Porter (1988) has suggested that the term "direct familiarity" be used to denote learning about kin by association with them and that "indirect familiarity" refer to the ability to recognize unfamiliar kin via phenotype matching. Unfortunately, Waldman (1988) has used the same terms to describe other types of cues. By his definition, " direct recognition" means that kin use cues expressed by conspecifics such as chemical cues or vocalizations to recognize another individual whereas "indirect recognition" would refer to the use of contextual cues not directly presented by an individual such as a parent

recognizing an offspring because it is located in the nest. The following review of the literature provides evidence that, in rodents, odours may play a role in such kin recognition contexts as parent-offspring and sibling recognition and mate choice.

2.4.1. Parent-offspring recognition

While the ability of fathers to recognize their own young has occasionally been described (Mallory & Brooks, 1978; Ostermayer & Elwood, 1983; Makin & Porter, 1984), mother-offspring recognition has been reported more frequently (Holmes, 1990). Since the production and care of young is critical to female reproductive success, this sex difference in recognition behaviour should not be unexpected (Trivers, 1972). Moreover, since males often leave the female soon after mating, they generally would not have the opportunity to interact with their own or other offspring. The ability of males from a biparental species such as California mice (*Peromyscus californicus*) to discriminate their young from the offspring of others has not yet been examined.

The ability of female rats to use olfactory cues to discriminate between their own young and other young of the same age was first reported by Beach & Jaynes (1956). When given the opportunity to retrieve alien or own young in an open field test, the mothers consistently rejected the alien pup, although by the end of the test session, they had also carried it to the nest. Most significantly, after their olfactory bulbs were destroyed, the subjects no longer discriminated between the pups, thus, confirming a role for odours in the discrimination process. Subsequent to this discovery, many studies have revealed that olfaction is critical for the onset and maintenance of maternal behaviour (Kresnegor & Bridges, 1990).

The use of odour-based pup recognition cues by mothers has also been demonstrated in guinea pigs (Porter, Fullerton, & Berryman, 1973) and flying squirrels (*Glaucomys*

volans) (Muul, 1970) but not in spiny mice (*Acomys cahirinus*) (Porter & Doane, 1978). Ostermayer and Elwood (1983) have pointed out that ultrasonic vocalizations may also be a source of discriminable cues. To test this hypothesis, they recorded the number of vocalizations of mouse pups while adults were given serial presentations of own and alien pups in a habituation-dishabituation paradigm. Both males and females initially showed a high sniffing response toward their own pups which diminished with repeated presentations of familiar pups. When an alien pup was presented, the levels of investigation increased for both males and females. The quantity of ultrasounds did not differ, which suggests that such vocalizations were not providing discriminable cues. Support for the hypothesis that ultrasonic vocalizations are not necessary for mothers to interact with their pups comes from the finding that genetically deaf mice show no differences in maternal behaviour from normal mothers (D'amato & Populin, 1987).

The most extensive studies of mother-offspring recognition have been undertaken in two species of ground squirrels: Richardson's ground squirrel (*Spermophilus richardsonii*) and Belding's ground squirrel (*S. beldingi*). Michener (1971, 1974) has traced the development of the discrimination process in Richardson's ground squirrel. Initially, mothers retrieved their own and other pups indiscriminately. By the time the young were 20-24 days old, the proportion of body and care-giving contacts between unrelated pairs decreased whereas the proportion of agonistic contacts increased (Michener, 1974). The role of olfactory cues in identification was not tested directly, although it was noted that there was a high incidence of nasal contacts among both related and unrelated squirrels (Michener & Sheppard, 1972).

Both field and laboratory tests have revealed that *S. beldingi* mothers discriminated between their own and alien offspring but only if the non-kin young were unfamiliar. In a field study, alien young which were placed near natal burrows and retrieved, were treated

as offspring whereas young reared by other females were treated aggressively (Holmes & Sherman, 1982). The dams only accepted new pups that were less than 25 days old. Similar results were obtained when cross-fostering experiments were carried out in a lab setting (Holmes, 1984). Confirmation that olfactory cues were used in the recognition process was provided by Holmes (1984a), who demonstrated that dam-offspring recognition as measured by lack of aggressive encounters no longer occurred after the subjects were "olfactorily impaired" with intranasal injections of zinc sulfate.

Holmes (1990) emphasized the importance of using different behavioural measures to assess discrimination abilities. In a comparison of previously collected data, he found that, in field tests, 15 day old cross-fostered pups were retrieved equally as fast as true kin whereas unfamiliar young were not (Holmes, 1984a). In contrast, when the same young were placed in the mother's home cage in the laboratory, she sniffed unfamiliar young for a longer period (Holmes & Sherman, 1982). It is possible that different recognition cues were used in the two different environments and as Holmes (1990) pointed out, one must attempt to design ecologically appropriate lab experiments and be judicious in one's interpretation of the results.

2.4.2. Offspring-parent recognition

Offspring-father recognition has not often been examined (but see Hepper, 1986b), although rat pups raised with both parents have been shown to be attracted to the feces of their father compared to the feces of other fathers or unmated males (Brown & Elrick, 1983). Holmes (1990) has suggested that pups benefit from recognizing their parents because it increases the likelihood that parents will recognize them. In addition, parents may be in conflict with their offspring with regards to how long and how much parental

investment is necessary (Trivers, 1974). If this is so, it would also be important for young to recognize their parents in order to maintain their own fitness.

A number of studies have shown that rat pups could discriminate between their own mother and a virgin female. Nyakas and Endroczi (1970) used a U-maze to show that 10 day old rats approached a goal box containing their own mother more often than a box housing a non-lactating females. Although whole animals were placed in the goal boxes, the random behaviour of the rats after the application of a local anesthetic to their noses would suggest that odour based cues were used for discrimination. Nonetheless, visual and auditory cues were still available in this paradigm.

A more appropriate system was developed by Leon and Moltz (1971) in which the goal boxes were separated visually from the main arena. Filtered air provided a masking noise as it passed through the goal boxes and carried the odours across an open area and into the start box. Thus, both visual and auditory cues were eliminated. In this study, 16 day old Wistar rat pups were able to discriminate between the whole body odours of their own mother and a nulliparous female. As the pups could discriminate between the odours left in the goal boxes after the adults had been removed, non-odour cues were eliminated as a possible source of influence.

The odours of a strange lactating female and a nulliparous female were discriminable but the odours of their own mother and those of another lactating female were not discriminable. Consequently, the authors hypothesized that a maternal pheromone exists which serves to identify a lactating mother (Leon & Moltz, 1971). Using a similar apparatus, a number of investigators found similar results in a different strain of rats (Holinka & Carlson, 1976) as well as in house mice (Breen & Leshner, 1977) and spiny mice (Porter & Ruttle, 1975; Porter & Doane, 1976; Porter, Doane & Cavallero, 1978).

In addition, guinea pigs (Porter, Fullerton, & Berryman, 1973) and gerbils (Gerling & Yahr, 1981; Yahr & Anderson-Mitchell, 1983) also spent more time near maternal whole body or nest odours compared with the odours of virgin females when tested in a two-choice discrimination box. Also, infant rats separated from their mother had a decrease in heart rate which was ameliorated by the presence of the maternal odour but not by the odour from a non-lactating rat (Compton, Koch, & Arnold, 1977). In these latter reports, the maternal pheromone was shown to provide a rather non-specific cue; as such, it could not constitute an odour of individuality. Nonetheless, the odour would constitute part of the overall odour profile of a lactating female.

Hepper (1986b) examined the investigatory response of rat pups to the odours of their own mother and an unfamiliar lactating female in a T-maze olfactometer. The pups which were tested daily between the ages of 5 and 22 days were separated from their mother for 30 minutes prior to testing. Under these conditions, pups crawled toward the goal box containing their mother. Consequently, Hepper concluded that rat pups can recognize their own mothers.

Hepper (1986b) pointed out that procedural differences could account for the difference between his results and those of Leon and Moltz (1971). Most significantly, he only removed the pups 30 minutes prior to testing, whereas Leon and Motz (1971), Holinka and Carlson (1976), Breen and Leshner (1977), Gerling and Yahr (1981) and Yahr and Anderson-Mitchell (1983) had separated their test subjects for several hours prior to the experimental procedure. Lack of food and warmth resulting from the latter procedure could have eliminated any discriminatory behaviour in these pups.

Using the same apparatus and procedure, Hepper (1986b) also determined that pups could recognize their fathers. Pups crawled toward their father at the start of the experiment but by Day 18, they were attracted to the odours of alien fathers. This reversal

in investigation time with increasing age is similar to that shown by siblings for own sibling versus unfamiliar age mate (Hepper, 1983).

In the former experiment (Hepper 1986b), the pups had been raised with both parents and it seemed possible that maternal labelling could have accounted for the offspring-father recognition. This possibility was eliminated by showing that pups could also discriminate between the odours of fathers which were removed from the cage prior to the birth of the pups and the odours of alien fathers (Hepper, 1986b). This result has provided one of the few experimental demonstrations of discrimination between unfamiliar kin and unfamiliar non-kin, possibly as a result of phenotype matching.

2.4.3. Sibling recognition

The study of the odour discrimination process among rodent siblings has contributed greatly to our understanding of the ontogeny of kin recognition. In general, results have indicated that recognition occurs by familiarity as well as through the process of phenotype matching (Blaustein, Bekoff, & Daniels, 1987). Many of these studies have directly examined the contribution of environmental and genetic components of odours to the recognition process (Doane & Porter, 1978; Porter, Matochik, & Makin, 1983); some have only implied that odours provide the necessary cues (Gavish, Hofmann, & Getz, 1984; Caley & Boutin, 1987) and still others have focused upon the role of non-odour cues (Schwagmeyer, 1980).

2.4.3(a) Spiny mouse (*Acomys caharinus*): Porter and his colleagues (reviewed in Porter, 1988) undertook a comprehensive examination of the development of sibling recognition in the precocial spiny mouse. In their studies, they provided mice with various types of social experience and then removed the mice from their home cage and examined the dyadic associations among them in an observation chamber. If mice huddled more

frequently with specific individuals, this behaviour was taken as indicative of discrimination. The role of olfactory cues in the recognition process was established by determining that mice which were made anosmic no longer showed huddling preferences (Porter, Wyrick & Pankey, 1978; Porter, Matochik, & Makin, 1986).

Their initial experiments showed that littermates preferred to associate with each other rather than with unfamiliar non-siblings; however, this preference did not occur if the subjects were housed together with the non-siblings for the five days prior to testing (Porter, Wyrick & Pankey, 1978). Moreover, mice that were isolated from their siblings for five days still retained their huddling preferences whereas those isolated for eight days did not (Porter & Wyrick, 1979). A four hour re-exposure period was sufficient to reinstate sibling preference. As mice that were separated from their littermates and housed with one littermate for nine days also huddled with an "unfamiliar" littermate after this period, Porter, Matochik and Makin (1983) suggested that odour discrimination was a result of phenotype matching.

Cross-fostering techniques were used to further examine the role of experience in sibling recognition. Pups which were cross-fostered on the day of birth or 10 or 20 days after birth all preferred to huddle with their foster littermates rather than with biological siblings or unrelated, unfamiliar weanlings (Porter, Tepper & White, 1981). Moreover, maternal labelling was shown to influence recognition ability. Both siblings and non-siblings reared apart but nursed by the same lactating female associated with each other significantly more often than either siblings or non-siblings nursed by different females. Nonetheless, it appears that exposure to littermates had some influence on recognition as non-sibling pups that were exposed to each other and to a common lactating female associated more frequently than those with only maternal exposure (Porter, Matochik & Makin, 1984).

These results along with those described above (Porter et al., 1983) would suggest that recognition cues have both genetically and environmentally mediated components. More recently, Porter, Matochik and Makin (1986) tested the ability of weaned mice to discriminate between siblings with whom they had associated for 10 days and other siblings from whom they had been separated for 10 days. Siblings which were raised together paired more frequently with each other than with their unfamiliar siblings. Porter et al. (1986) concluded that interactions among siblings are a result of recognition of individual signatures. It appears to me that the odours of the discriminating individuals themselves could have been associated with the familiar conspecifics, thereby providing a recognition cue.

The development of responses of young spiny mice to odours of conspecifics was also studied by Janus (1988). He examined the responses of siblings to the bedding odours of parents and siblings rather than only to siblings but, nonetheless, he raised specific points which are relevant to such research. He pointed out that the two choice test used by Porter's group allowed for assessment of preference but not attraction. The inclusion of two arenas with clean bedding odour and a third with no bedding enabled the relative preference or aversion to two odours to be examined simultaneously. While it might be argued that the third chamber was no different than the neutral area of the main arena, the author suggested that the neutral enclosure was more attractive because it was small and dark.

The results of Janus's (1988) study indicated that 1-10 day old pups were attracted to the odours of siblings and parents but not to those of unrelated adults. This preference changed as the pups grew older and by 25 days of age, the pups preferred the odours of unfamiliar, unrelated adults. Thus, the findings regarding the young pups agreed with those of Porter, Doane and Cavallaro (1978). With the multi-choice testing apparatus,

Janus (1988) was able to show that 20 day pups were attracted to the odours of both lactating and non-lactating females whereas in his two odour comparison, Porter et al., (1978) showed that lactating odour was still preferred. Thus, it appears that the design of the study can influence its outcome and care should be taken when generalizing results based upon only one paradigm.

2.4.3(b) Deer mouse *Peromyscus leucopus*: The somewhat conflicting findings reported in the study of sibling interaction in white-footed mice may also have been a result of differences in methodology. These investigations were concerned with the mechanism by which recognition occurred. Grau (1982) stated that huddling preferences depended on both familiarity and phenotype matching. Mice huddled with familiar individuals whether non-sibling cagemates or littermates but non-littermate siblings were also discriminated from non-littermate non-siblings. In this latter instance, although non-littermate siblings were from different litters, they still experienced the same uterine environment so it is still possible that familiarity with odours is relevant to this discrimination.

Halpin and Hoffman (1987) found that there were no significant differences in time spent with siblings reared apart and unfamiliar non-siblings. They concluded that familiarity was the only mechanism involved in sibling recognition in white-footed mice. The authors suggest that methodological differences related to the age of cross-fostering and time spent associating with littermates and the way recognition was measured may have resulted in the discrepancy between their study and that of Grau (1982). This issue remains unresolved. To avoid the possibility that the uterine environment creates a degree of familiarity among non-littermate and littermate siblings, it would be appropriate to use paternal non-littermate siblings as a basis for comparison in such an experiment.

2.4.3(c) Voles (*Microtus*) Another factor which should be considered when studying the development of recognition behaviour is the social structure of the species. Wilson

(1982) has done so by comparing sibling interactions in two species of voles: the prairie vole (*Microtus ochrogaster*) and the meadow vole (*Microtus pennsylvanicus*). The prairie vole is highly social (Getz & Carter, 1980) whereas the meadow vole is not (Krebs, 1979). As Wilson (1982) had hypothesized, the prairie voles associated with conspecifics more frequently than did the meadow voles. In most instances of social contact, non-siblings were investigated more than siblings in both species although the magnitude of the difference was greater in prairie voles. The meadow voles showed no difference in the amount of "neck nosing". The significance of this finding is unknown as the biological role of sniffing different body parts was not made clear. Nonetheless, this result does emphasize the value of measuring many facets of behaviour when assessing discriminability of individuals. In a similar species, both male and female common voles (*Microtus arvalis*) preferred the odours of unfamiliar non-siblings to those of familiar siblings (Bolhuis, Strijkstra, Moor, & van der Lende, 1988). Thus, odour familiarity seems to be a predominant factor in recognition in voles.

2.4.3(d) The house mouse (*Mus musculus*): The influence of self-odours and those of littermates and parents in the odour recognition process has been examined in laboratory mice. Kareem and his colleagues assessed a number of investigatory and aggressive behaviours in juvenile and adult males and females in an open field arena. In their initial experiment, they determined that adult male paternal half-siblings sniffed and investigated each other less than unfamiliar non-siblings but more than full siblings. The time spent investigating by adult females did not differ between half-siblings and non-siblings. Juveniles of both sexes showed the same trends as adults except that all juveniles engaged in more investigatory behaviours than adults (Kareem & Barnard, 1982). To determine the role of familiarity in these findings, the authors examined the behaviour of the following males: unfamiliar non-siblings, unfamiliar half-siblings, familiar non-siblings, familiar

half-siblings and full siblings. Pairs of familiar animals investigated each other equally; however, in the unfamiliar groups, scores of half-siblings were intermediate between those of non-siblings and full-siblings. These findings suggest that genetic cues can be used to discriminate degrees of relatedness but that familiarity offsets this effect.

The effects of different amounts of post-natal familiarity on sibling recognition were also examined (Kareem, 1983). In a unique design, males were cross-fostered into groups composed of both siblings and non-siblings from birth, day 7, 14, 21, 30 or 50. Additional groups were cross-fostered or reared by their own parents until weaning and then housed with their siblings until testing (70 days of exposure). There was a negative correlation between exposure and length of investigation. Unfamiliar siblings, separated on the day of birth, socially investigated, i.e., sniffed, nosed, investigated and groomed, each other more than did siblings reared together for 21, 30 , 50 or 70 days. Their behaviour did not differ from subjects with 7 or 14 days of familiarity. This may mean that with less than 21 days of experience, and experience with other individuals, mice do not remember their siblings. There were also differences in overall activity among unfamiliar non-siblings compared with both familiar and unfamiliar siblings. It seems possible that unfamiliar siblings had a vicarious familiarity with each other as they had been raised with other siblings from the same litter.

A number of investigations of sibling recognition in mice have examined the influence of such factors as age, sex and the presence of parents upon the discrimination process (Gilder & Slater, 1978; D'Udine & Partridge, 1981; Hayashi & Kimura, 1983). Kareem and Barnard (1986) examined the effects of all of the above factors in single and mixed sex pairs of adult-juvenile mice by investigating the social relationships between full siblings, maternal half-siblings, paternal half-siblings and non-siblings. In single sex pairs, there were no differences in investigation of any sibling groups and only males showed any

differences between siblings and non-siblings. In mixed sex pairings, recognition depended upon the relative ages of the males and females. Adult male-juvenile female pairs discriminated between full and half siblings and paternal and maternal half siblings but adult female-juvenile males did not discriminate among their conspecifics in any circumstance. Thus, a maternal presence appeared to affect male-female interactions only in a very specific context. In general, the results of this study contrast with the findings of Kareem and Barnard (1982) which indicated that same age and sex juveniles could discriminate among kin and non-kin. The authors suggested that odours change with age but the possibility that differences in levels of motivation are responsible for the discrepancy should also be considered. It is important to remember that lack of discrimination is not synonymous with lack of recognition.

To assess the relative importance of self and familiar phenotypes in male mice, Aldous (1989) cross-fostered single individuals into a litter of non-relatives; the remainder of their siblings were raised with their own mother. He then measured the investigatory behaviour of both the cross-fostered subject and their non-fostered littermates as juveniles and as adults to their unfamiliar paternal siblings, the paternal siblings of the foster sibling and unfamiliar non-kin.

He found that juvenile cross-fostered and non-fostered subjects discriminated between non-kin and their own paternal siblings and the paternal siblings of their foster siblings but not between the two sibling groups. Cross-fostered subjects were touched more by both their own relatives and their foster-littermates relatives than by non-kin. Both results suggest that the cues of a familiar littermate, whether related or not, influenced the discrimination of their relatives. As adults, neither non-fostered nor cross-fostered subjects discriminated among kin, non-kin or foster-kin. Among the stimulus animals, unfamiliar siblings and foster-kin siblings discriminated their own unfamiliar non-fostered

kin and kin of the fostered littermates from non-kin but did not discriminate between the latter two groups. Thus, adults appear to be less motivated to discriminate among individuals in the conditions employed in this experiment. This finding is puzzling and not readily explicable.

In laboratory mice, more than in any other species investigated, the results often appear to be incongruous. Of course, mice are also the most frequently investigated species and thus, the results provide more opportunity for comparison and subsequent contradiction. The experiments were frequently conducted with different strains of mice of different ages; moreover, the methodology often varied. The adoption of consistent experimental procedures may resolve some of the discrepancy in results.

2.4.3(e) The rat (*Rattus norvegicus*): Several investigators have examined the responses of rats to the odours of their siblings. Using a T-maze, Hepper (1983) found that rat pups could discriminate between whole body odours of unfamiliar related siblings and unfamiliar unrelated non-siblings. The animals preferred their siblings during testing at 10-14 days of age, but switched their preference to unrelated individuals at 18-22 days of age. As the "unfamiliar" siblings were separated two or four days postnatally, Hepper suggested that no postnatal influence remained when the animal's preference was tested. He concluded that phenotype matching, learned prenatally, was likely involved in the odour discrimination of kin.

He supported his argument by citing Stickrod, Kimble and Smotherman's (1982) finding that rats could acquire a learned taste aversion prenatally. He did not note that Rudy and Cheatle (1977) have shown that two day old rat pups are also capable of associative learning and that they retain this learning when tested at ten days of age. Thus, it is possible that the unfamiliar siblings removed at two days of age in Hepper's study did have a postnatal influence.

Wills, Wesley, Sisemore, Anderson and Banks (1983) examined the response of rats to bedding odours only and confirmed Hepper's finding that odours of unfamiliar, unrelated agemates were preferred to those of "unfamiliar" siblings by albino rat pups ranging in age from 25 to 35 days. Wills et al. (1983) also found that rat pups showed no preference for odours of familiar non-siblings over familiar siblings. The familiar siblings and non-siblings were reared with the subject and could possibly be "labelled" with the subjects own odour and/or the odour of their mother. If either of these odour cues are commonly used to discriminate between individuals, it could explain the inability of rat pups to discriminate between the two unrelated odour donors which shared the same rearing conditions as the subject.

Most interesting was the demonstration that the subjects showed no preference for odours of unfamiliar siblings over familiar non-siblings despite the obvious familiar-unfamiliar dichotomy (Wills et al., 1983). One explanation is that the unfamiliar sibling and familiar non-sibling are both labelled with the subject's own odour or their common maternal odour; however, this seems unlikely as the test date was about 30 days after the subject and unfamiliar sibling were separated. An alternative explanation is that a recognition memory for phenotypic match of own sibling may be equated with the familiarity of the odour of the cross-fostered sibling. Such an interpretation suggests that familiarity of odours and phenotype matching by odour may both contribute to the basis of odour discrimination.

Using the odours of juvenile sibling rats as stimuli, Hopp, Owren and Marion (1985) provided conclusive evidence that rats are able to discriminate not only between siblings and non-siblings but also between the odours of individual siblings. Using a Y-maze, they initially trained juvenile male rats to discriminate between the odour of a sibling (S+) and no odour (S-). Then, the subject was given a choice between the S+ sibling and an S-

sibling. The subject was immediately able to make this discrimination. Hopp et al. (1985) suggest that this level of performance indicates that the ability to discriminate between siblings requires no training.

Since the odours of littermates were used as stimuli, degree of familiarity and relatedness were eliminated as possible confounds in this study. Other possible differential cues such as age, sex and group odours and familiarity were also held constant. Although dominance status was not controlled, the odours were distributed as positive and negative stimuli in such a manner as to prevent social status from being used as a discriminatory cue. Consequently, experimental use of this procedure provides a most convincing argument that odours of a constant nature can be used for individual recognition.

The authors speculated that the differences in odours could arise from dietary or genetic sources. It is also possible that differences in intrauterine environment could affect the discriminability of siblings. Female Mongolian gerbils which develop between male fetuses are androgenized and as a result develop patterns of eye opening and paw preference dissimilar from other females; moreover, some characteristics of these gerbil dams are genetically transmitted to their daughters (Clark, Robertson, & Galef, 1993; Clark, Karpiuk, & Galef, 1994). While it has not been demonstrated that the odours of individual littermates could also be influenced by androgenization, it seems possible that such is the case, given that androgen levels affect odours of dominance.

The ability of rats to discriminate between siblings and relatives of specific degrees of relatedness was examined by Hepper (1987b). He tested the odour preference of juvenile male and female rats for the following same-sex age-mates: 1) "unfamiliar" sibling, i.e., cross-fostered with another litter from two days of age; 2) unfamiliar paternal half-sibling; 3) unfamiliar first cousin and 4) unfamiliar unrelated individual. Male and female rats were presented simultaneously with stimulus rats from all four groups in wire mesh cages.

Significantly more time was spent investigating the odours of unrelated individuals followed in decreasing order by the odours of first cousins > half-siblings > full siblings. While degree of familiarity is different for the full sibling compared with the other individuals, there were no known differences among the other groups. With this one exception, such results provide conclusive evidence not only that detectable differences are present in the odours of individuals but also that these differences may be used to discriminate among equally unfamiliar kin of different degrees of relatedness. As other factors were held constant, Hepper (1987a) stated that these results indicate that a genetic cue is involved in the odour identification process.

As has been demonstrated throughout this review, rodent young may learn the cues of recognition within the nest environment. Hepper (1987b) examined the relative contribution of siblings and their mother to recognition of kin. He scented mother and siblings differentially with perfume odours and examined the preference of 12 and 20 day-old pups for samples of the perfumes in a T-maze. Pups preferred the odours of their siblings. The mother's odour was preferred to an unfamiliar perfume, thus, the previous results could not be attributed to aversion to the odour associated with the mother. While the pups may have been exhibiting a preference for the odours of littermates with whom they associate more frequently than the mother, they may also have been navigating toward an even more familiar odour, the odour of themselves.

2.4.3(f) Squirrels (*Spermophilus*): An examination of dyadic interactions in juvenile ground squirrels lends support to the theories that both familiarity with an odour and matching the odour to a template based upon its phenotypic characteristics may account for discrimination in this species. For example, an analysis of social encounters in Richardson's ground squirrels revealed that siblings reared apart and siblings reared together treated each other similarly and that non-siblings reared together or apart were also

not discriminated (Davis, 1982). Siblings and non-siblings reared apart were treated differently. The squirrels were cross-fostered at Day 1, separated and housed individually at Day 37 and tested at approximately 110 days of age so it seems likely that the odours of kin were recognized on the basis of template matching and not because of prior association.

In contrast, pairs of thirteen-lined ground squirrels (*S. tridecemlineatus*) reared together and tested at about 45 days of age explored each other equally regardless of whether or not they were related (Holmes, 1984b). Furthermore, unrelated and related pups that were reared apart also showed no difference in levels of exploration. Thus, it appears that recognition is based upon odour familiarity only. After the animals were made anosmic with zinc sulfate, the amount of exploratory behaviour decreased in all animals, thus, confirming the importance of olfactory cues in behavioural preferences. These results contrast with those of Davis (1982) but the two species differ in their social structure, with thirteen-lined squirrels being non-social. Moreover, the subjects were tested at different ages. It would be interesting to examine the behaviour of such pairs at a later date to determine if the same cues are still used as the subjects become less familiar with each other.

Studies of Belding's ground squirrels and Arctic ground squirrels (*S. parryii*) in the lab indicated that non-siblings reared together and siblings reared together were significantly less aggressive than either group reared apart. Sisters reared apart were significantly less aggressive than unrelated females but brothers and brother-sister pairs were not less aggressive (Holmes & Sherman, 1982). As well, full sisters fought less often and were more co-operative than were half-sisters. Thus, it would appear that the odours of individuals must be compared with a particular template for recognition.

Confirmation for this hypothesis comes from laboratory observations of paired encounter tests with female Belding's ground squirrels (Holmes, 1986a). A number of

pups from each of four litters were cross-fostered so that siblings and non-siblings were exposed to siblings and non-siblings of those encountered in the test situation. The results indicated that such pre-exposure reduced exposure in females compared to females with no pre-exposure, whether the pairs were related or not. Moreover, sisters indirectly exposed to each other were less agonistic than non-sisters indirectly exposed to each other. Holmes concluded that females learned the phenotypic characteristics of their nestmates as well as of themselves and that both could influence their ability to discriminate their conspecifics.

A further investigation of the ability of yearling Belding's ground squirrels to identify familiar siblings, unfamiliar paternal half-siblings, and unfamiliar unrelated conspecifics demonstrated that same sex or mixed sex pairs of siblings were less agonistic than pairs of half-siblings or unrelated individuals. Furthermore, female half siblings were less agonistic than unrelated pairs (Holmes, 1986b). While Holmes cautions against extrapolating from laboratory data to field conditions, he did suggest that the social habits of females may have lead to the evolution of such mechanisms.

These latter investigations as well as those studies of odour discrimination in rats of different degrees of relatedness (Hepper, 1986b; 1987) have conclusively demonstrated that discrimination of kin can be based upon knowledge of phenotypic cues which is not learned by prior association.

2.4.4. Discrimination between kin during mate choice

A number of attempts have been made to test Bateson's (1983) optimal outbreeding theory by examining the choice of animals to mate with related or unrelated individuals of the opposite sex. Some investigators have specifically analyzed the preference of individuals for the odours of opposite sex individuals. Such studies have been criticized because they have not examined copulation as part of the test procedure (Manning, Potts,

Wakeland, & Dewsbury, 1992). Recently, one investigator has assessed both reproductive success with particular mates and odour preference of similar individuals in the same experiment (Keane, 1990). This latter study as well as others which have investigated odour preferences for potential mates will be reviewed here.

2.4.4 (a) Mate choice behaviour in inbred strains of mice: Extensive analyses of the mating preferences of both male and female house mice with regard to strain preference were made by Yanai and McClearn (1972a-c). These were among the first examinations of mate choice and the results were not consistent. Initial observations that females of two inbred strains (DBA/Ibg and C57BL/Ibg) associated and mated with males of the opposite strain (Yanai & McClearn, 1972a) were followed by an examination of mating behaviour in several more strains (Yanai & McClearn, 1972b). In the latter experiment, a technique was employed to minimize the potential for male interference by providing a tube-like opening for the females to investigate the male through without making physical contact. The results were variable, with the females of the BALB/Ibg and C3H/J strains not showing any preference and the preferences of DBA/Ibg and C57BL/Ibg being strain dependent, i.e., they preferred the opposite strains. Males of the DBA/Ibg and BALB/Ibg strains were tested and showed no preferences.

Finally, the mate choice of females of the BALB/Ibg and DBA/Ibg strains and the female offspring of reciprocal crosses of these strains raised with their biological mothers and fostered with fathers of the original strains was examined (Yanai & McClearn, 1972c). Again the results were strain dependent, with one strain of females showing preferences for the males of the other strain. Pups with foster fathers of this strain preferred males of their own strain, which the authors suggest reflects a negative "imprinting" process.

Gilder and Slater (1978) directly examined the interest of 10 week-old mice in the odours of their siblings and non-siblings of the same and different strains (Porter homozygous normal nad Steel homozygous normal). Females preferred the odours of a non-sibling male of the same strain to the odours of their brothers and preferred their brother's odours to those of a male of a different strain. Males did not show significant preferences for the odours of any of the females although there was a trend for greater investigation of individuals less closely related to them.

In an examination of the role of familiarity in mate choice, D'Udine and Partridge (1981) compared the odour preferences of adult male and female mice from two inbred strains (C57 and SEC). All mice were reared by foster parents of their own strain (in-fostered) or by foster parents of another strain (cross-fostered). The preferences of males suggest that familiarity rather than strain or kin differences contribute to choice of odours. In-fostered males from both strains preferred the bedding odours of a non-sibling to their littermate sibling but cross-fostered males showed a preference for odours of their unfamiliar natural sibling rather than for their familiar littermate sibling of a different strain.

In contrast, if females made a choice, it was based upon relatedness. C57 females preferred their siblings regardless of their familiarity. Females of the SEC strain showed no significant preference. The inconsistencies among these results and those of Yanai and McClearn (1972a-c) and Gilder and Slater (1978) may be reflective of the concerns raised by Manning, Potts, Wakeland & Dewsbury (1992) regarding the use of inbred strains in mate choice experiments. They have suggested that the inbreeding process imposed artificial selection pressures which may dramaticlly alter mate choice behaviour. Moreover, in these studies, the estrous state of the females was not controlled. This may also have influenced the outcome of the choice tests.

2.4.4 (b) Mate choice among siblings and individuals of other degrees of relatedness: Discrimination of siblings from non-siblings has been examined in the context of mate choice in several species of rodents: cactus mice (*Peromyscus eremicus*), deer mice (*P. maniculatus*), voles, house mice and gerbils. In general, the findings support a role for odours of familiarity in providing recognition cues. Cactus mice breed more frequently with non-siblings than siblings except when the non-siblings had been cross-fostered. In addition, when siblings were isolated from each other the avoidance of incestuous breeding declined (Dewsbury, 1982b). Moreover, deer mice did not show a reduction in the number of litters produced whether they were mated with siblings or non-siblings (Dewsbury, 1982b). The time at which the mice were paired may influence litter production, however, as Hill (1974) found that pairing siblings as well as familiar non-siblings caused delayed reproduction if the mice were paired prior to puberty.

An examination of the preference of adult outbred lab mice in a two-way choice apparatus revealed that adult males preferred unrelated, unfamiliar females to their sisters or mothers and preferred unfamiliar related females to unfamiliar unrelated females (Hayashi & Kimura, 1983). In this paradigm, females did not show any preference. A study of adult female house mice given the opportunity to make sensory contact only with littermate siblings, unfamiliar full siblings of a prior or subsequent litter or unfamiliar non-siblings in a paired choice test revealed that these females spent more time with unfamiliar non-siblings than with individuals of the other groups (Winn & Vestal, 1986). They also associated more with familiar rather than unfamiliar siblings. This latter study is one of only several which has found that mice recognize unfamiliar siblings.

An examination of mating behavior in prairie voles revealed that non-siblings raised as littermates avoided breeding whereas siblings separated for eight days showed no evidence of incest (Gavish, Hoffman & Getz, 1984). Similarly, pairs of gray-tailed voles

raised together produced fewer litters than unfamiliar pairs regardless of whether they were siblings or non-siblings (Boyd & Blaustein, 1985). In this case, individuals that were separated for 5-12 days from littermates with whom they were reared still did not mate when they were reunited.

The variables influencing incest avoidance have also been examined in gerbils and again the results indicate that familiarity with a potential mate prior to puberty causes a delay in mating regardless of the genetic relationship between the individuals (Agren, 1981; 1984). For gerbils, it has been demonstrated that saliva appears to provide the cues necessary to discriminate among individuals (Block, Volpe & Hayes, 1981). The particular salivary compounds which might be responsible for this discrimination have not been identified.

Recently, several efforts have been made to determine the preference of mice for the odours of opposite-sex individuals or the individuals themselves which differ in degrees of relatedness. In an attempt to determine the role of kinship and familiarity in mate choice, Barnard and Fitzsimmons (1988) examined the preference of sexually mature, cross-fostered mice for the soiled bedding odours of conspecifics of five degrees of relatedness. In one test, the stimulus odours were familiar as the subjects shared a cage with each other but were separated by a perforated partition; in the other test, all odours were from unfamiliar individuals except for the odour from their own sibling which the subject had been exposed to prenatally and postnatally prior to cross-fostering.

The results were influenced both by the sex of the test subject and their familiarity with the odour donor. Neither males nor females showed any preference for odours of unfamiliar individuals regardless of their degree of relatedness; nor did females show a preference for any of the familiar odours. The males discriminated among some of the familiar odours, with both familiar siblings and half siblings being preferred to second

cousins and unrelated individuals. The authors suggested that the males may have only discriminated between familiar animals to which they were likely to be related. While the females showed a lack of odour preference, they were attracted to full and half siblings rather than first or second cousins or unrelated conspecifics in a freely mixing group. The contradiction between investigation of odours and investigation of animals may reflect the complexity of the relationship between odour preference and the presence and absence of the odour donor. Furthermore, the inconsistent nature of the results in this study and the previous reports may also indicate that methodological issues should also be addressed in future examinations of the role of individual odour cues on mate choice in mice.

To assess the relationship between reproductive success and mate choice, Keane (1990) first bred pairs of white-footed mice which were full siblings, half siblings, first cousins and non-relatives and measured the offspring weight at birth and weaning. Generally, the offspring of non-relatives and first cousins were larger at birth and weaning. Subsequently, an examination of the number of times the estrous female F2 offspring of these pairs entered pairs of empty nestboxes of non-littermate siblings, first cousins and non-relatives indicated that they preferred the odour of their first cousin over a non-relative but showed no preference between siblings and first cousins or non-relatives. When the boxes contained males rather than odours, the number of times the females entered the boxes did not differ; however, they spent more time within the box containing the first cousin than the sibling. They also directed fewer attacks and more "amicable" behaviours toward cousins than siblings. Similarly, they attacked cousins less frequently than non-relatives.

A comparison of these findings with a study by Barnard and Fitzsimons (1989) in which the litters of breeding pairs of full sibling, half-sibling, first cousin, second cousin and unrelated house mice were examined to determine if there were any fitness

consequences of mating with kin of different degrees of relatedness confirms the general direction of the findings. Half-siblings, first and second cousins and unrelated mice had more pups than did siblings.

2.5. Discrimination of unfamiliar non-kin

As the previous studies have demonstrated, an individual's body odour can be influenced by genetic factors, age and sex as well as by reproductive state and dominance status. To investigate the role of genetic differences, a number of studies have attempted to hold constant as many of these other factors as possible while examining the ability of subjects to discriminate individually unique odours in a variety of species, including mice, gerbils, chipmunk, guinea pigs and rats. As the subjects are unrelated to the odour donors, the results confirm the ability of genetic cues to be used to discriminate conspecifics in a context other than kin discrimination. The following studies are illustrative of the methods used in assessing this concept.

Using a Y-maze olfactometer, Bowers and Alexander (1967) trained male and female house mice to discriminate between the odours of two male mice from the same inbred strain. The odour donors were unfamiliar and unrelated to the subjects and to each other. However, as the housing conditions were unreported, the possibility of a dominance relationship between the odour donors cannot be discounted. Moreover, if the odour donors were housed in groups rather than individually, the discrimination could have been based upon group or colony odours.

Adult male and female Eastern chipmunks (*Tamias striatus*), tested in a habituation-preference task, could detect individual differences in the soiled bedding of unfamiliar male and female conspecifics (Keevin, Halpin, & McCurdy, 1981). Wild male guinea pigs

could discriminate between individuals using the odours of the supracaudal, urine and perineal secretions of two familiar colony mates when tested in a similar habituation paradigm (Martin & Beauchamp, 1982).

Following a report by Dagg and Windsor (1971) that gerbils could discriminate between the ventral gland secretions of individual conspecifics, Halpin (1974), in a well-controlled study, used a habituation-preference test to show that male Mongolian gerbils could detect individual odour differences in the ventral gland secretions, urine and soiled bedding of two unfamiliar conspecifics who had been individually housed. Halpin's careful work provided firm evidence that odours may function to identify individuals rather than categories of animals. Using the same paradigm, Tang-Martinez, Mueller and Taylor (1993) found that female golden hamsters could discriminate between flank gland secretions, urine, feces and soiled bedding from individual males. Moreover, when given the opportunity to mate with a male whose odour was familiar, females produced significantly larger litters.

Using a habituation-dishabituation task, Brown (1988) examined the ability of male outbred rats to discriminate between pairs of outbred rats in the same hormonal state. Odours from pairs of intact and castrated males and from pairs of estrous/ proestrous, diestrous/metestrous and ovariectomized females were discriminable. Although minor differences in sex hormones could be used in discriminating the intact males and females, no such variability could account for the discrimination between the pairs of gonadectomized animals. The author suggested that variables such as hormone levels do not contribute to individual odour production; rather, an odour reflecting an unchanging genetic component may be responsible for the production of individual odours in rats.

2.6. Summary

In this chapter, I have reviewed the studies which investigated the ability of rodents to discriminate between the odours of their conspecifics. The discrimination of odours related to reproductive state in females and dominance status in males have illustrated that hormonal changes provide discriminable cues and emphasized the importance of odours in influencing social behaviour. The reports of odour-based kin recognition have demonstrated that kin are discriminated from non-kin as a result of phenotype matching; nonetheless, it has also been demonstrated that discrimination based upon familiarity may be the predominant factor in determining which conspecifics are likely to be considered as "kin".

It seems likely that differences in hormonal metabolites provide the basis for the odour discrimination of estrous state in females and dominance status in males; however, the genetic basis of odours of individuality and the mechanism by which these odour cues are expressed remains uncertain. The one genetic region which has been investigated in this context is the major histocompatibility complex (MHC). Chapter 3 provides an overview of the molecular genetics of the MHC and describes how it may function in the production of odours of individuality.

CHAPTER 3

THE GENETIC BASIS OF ODOURS OF INDIVIDUALITY

The mammalian genome is comprised of multiple unique transcripts. The products of any number of these could provide a unique signal of individuality. While the results from a number of studies have indicated that recognition of individuals in a diversity of species can be based solely upon genetically determined cues (e.g. Holmes, 1984b; O'Hara & Blaustein, 1985; Smith & Wenzel, 1988; Linsenmair, 1987), the exact nature of these cues has seldom been examined. Nonetheless, one genetic structure, the major histocompatibility complex (MHC) has been consistently implicated in the production of olfactory signals (Boyse, Beauchamp, Yamazaki, & Bard, 1991). A number of behavioural studies (reviewed in Chapter 4) have revealed that differences in the genes of the MHC provide sufficient cues for mice and rats to discriminate between the urine odours of their conspecifics.

Other investigations have implicated the sex chromosomes in the production of unique odours (Yamazaki et al., 1986; Schellinck, Monahan, Maxson, & Brown, 1993). Genes closely associated with the MHC also appear to contain olfactory information relevant to mate choice (Lenington & Egid, 1985; 1989). The autosomal genes may also produce olfactory cues characteristic of an individual but the number of autosomes involved has not been determined (Boyse et al., 1991).

To understand how the MHC could be associated with the production of such odours requires some understanding of its basic structure and function. The review which follows summarizes the current level of knowledge of the MHC at the genetic, molecular

and cellular levels and discusses the way in which the MHC may control or alter odours of individuality.

3.1. Immunogenetics of the Major Histocompatibility Complex

The MHC is a cluster of genes that encodes for peptide binding molecules which present self and foreign peptides to cells of the immune system. Since potential self-reactive T cell clones are eliminated during differentiation, the presentation of self-peptides does not evoke an immune response (Kappler, Roehm & Marrack, 1987; Kappler, Starez, White, & Marrack, 1988). In contrast, the presentation of fragmented proteins which are derived from foreign antigens such as viruses, bacteria or parasites results in activation of the immune system. It is in the context of tissue transplantation that the MHC is most well-known. If the transplant is seen as foreign, it may be rejected by the host as a result of action of the MHC. This self-non-self discrimination by the MHC has been referred to as "kin recognition at the cellular level" (Brown & Eklund, 1994). The possibility that this expression of individuality at the cellular level could be translated into a behavioural signal was first suggested by Thomas (1974). He speculated that there may be a "biological relationship between the system of odorant labelling for the identification of self in humans, and the self-marking mechanism provided by the HLA complex of antigenic determinants" (pg 245). Subsequent operant conditioning experiments which demonstrated that the urine odours of both MHC congenic mice and rats are discriminable by conspecifics provided substantive evidence for this hypothesis (See Chapter 4).

3.1.1. The molecular organization of the MHC

MHC genes have been identified in many mammals as well as in a number of birds, fish and amphibia (Klein & Figueroa, 1986; Lawrance & Quaranta, 1991). Most of our knowledge regarding the MHC comes from genetic maps constructed for the MHC of the human, the mouse and the rat (Cortese Hassett, Stranick, Locker, Kunz, & Gill, 1986; Smith, Steinmetz, & Hood, 1986; Chimini & Pontarotti, 1991). Figure 3.1 provides a map of the genetic loci of the mouse and the rat which will be referred to in the following review of the structure and function of the MHC. The mouse MHC, known as the H-2, is on chromosome 17 (Klein et al., 1974). The MHC of the rat (RT1) was originally assigned to chromosome 14 (Oikawa et al., 1983) but has recently been shown to reside on chromosome 20 (Locker et al., 1990). The MHC of the human is referred to as the HLA and is located on chromosome 6 (Van Someran et al., 1974).

For a genetic region to confer knowledge about individuality, its nucleic acid code must be translated into a unique phenotypic signal which can be learned by conspecifics. Since an extraordinary number of alleles are expressed by the MHC genes in mice and humans, it is unlikely that, in an outbreeding population, any two individuals (except for identical twins) would have the same MHC genotype. For example, based upon our knowledge of differences in MHC loci in *Mus musculus*, it appears that 3.6×10^9 unique phenotypes could be coded (Singh, Brown, & Roser, 1987). Thus, it would seem that the MHC is a likely candidate for the production of a unique odour signal.

It should be noted that this polymorphism of alleles found within the MHC of mice and men is not as characteristic of the rat MHC (Gill, Kunz, Misra, & Cortese Hassett, 1987) so it is likely that the fewer phenotypes exist in rats than in mice (Klein & Figueroa, 1986). Despite this situation, odours of individuality have been demonstrated in rats which differ genetically only at specific MHC alleles. The variability in the number of genes

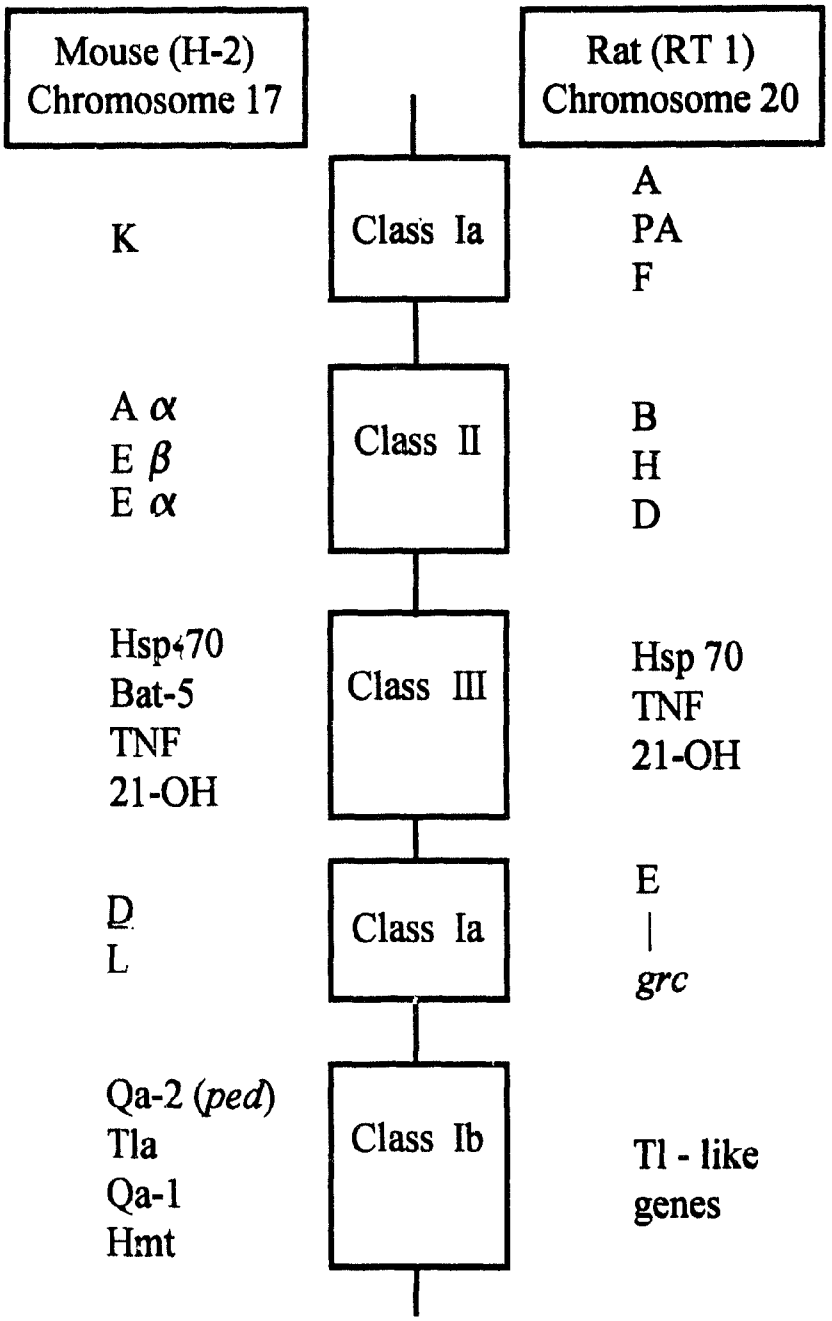


Figure 3.1: The organization of the genes of the MHC in mice (H-2) and rats (RT 1). Modified from Lawrance and Quaranta, 1991 and from Cortese Hassett et al., 1991.

expressed and their organization as well as limited polymorphism may contribute to the uniqueness of the MHC in some species. (Lawrance & Quaranta, 1991). It may be that, in rats, this combination of features is sufficient to confer signals of individuality.

3.1.2. Genes of the MHC

Three classes of major histocompatibility genes, class I, class II and class III, each with different structures and immune response functions, have been identified within the MHC. With each technological advance in molecular biology, more genes within the MHC complex are being identified and their functions investigated. Several genes which affect susceptibility to disease and others related to growth and development (such as the *grc* in the rat and the *ped* in the mouse) have been shown to be tightly linked to the MHC (Kunz, Gill, Dixon, Taylor, & Greiner, 1980; Warner, Gollnick, & Goldbard, 1987). In addition, the genes which encode the proteolytic machinery necessary for the formation of peptide-MHC units are expressed in association with the MHC genes. For example, the transporters associated with antigen presentation to the MHC molecule as well as the proteases which process antigenic peptides are MHC-linked and are also believed to be polymorphic (Spies et al.,1992).

The behavioural literature has focused mainly upon the potential role of the so-called classical class Ia genes in producing a unique phenotype. However, the odours of mice which are congenic for both nonclassical class I and class II regions of the MHC can also be discriminated (Yamazaki et al., 1982; Singh et al. 1987). These findings indicate that other MHC genes within the chromosome also contribute unique characteristics to an individual. The role of non-MHC genes which are linked to the complex in the production of individually unique characteristics remains largely unexplored.

3.1.3 Class Ia molecules

Class Ia genes code for cell-surface glycoprotein molecules which present antigenic peptides to T lymphocytes. In most tissues, hundreds of class Ia complexes are displayed on every cell (Elliott, Smith, Driscoll, & McMichael, 1993; Englehard, 1994). The level of expression varies, however, with high levels on lymphoid cells, lower levels in the liver and intestine, little expression in skeletal muscle and adrenals and almost none in the brain, sperm and embryonic tissues (Singer & Maguire, 1991). Each class Ia complex consists of three parts: an extracellular portion, a membrane spanning region and a 'cytoplasmic tail". The extracellular portion is divided into 3 domains specifically labelled 1, 2 and 3 which are noncovalently attached to a light $ß_2$ microglobulin chain (Bjorkman & Parham, 1990). The β2 chain is not encoded on the MHC chromosome and is invariant in structure (Lawrance & Quaranta, 1991).

The three-dimensional structure of the class Ia molecule of the HLA has been determined and provides the basis for understanding how the molecule binds specific peptides and interacts with "killer" T-cells (Bjorkman et al., 1987a, 1987b). The 3 and ß2 domains interact proximal to the membrane and provide support for the 1 and 2 domains. These latter two regions of the class Ia molecule interface in such a way as to provide a groove-like binding site for a foreign or self-peptide.

As illustrated in Figure 3.2, MHC molecules first become associated with a particular peptide during their synthesis on the endoplasmic reticulum. The peptides are derived from cellular proteins which have been degraded by intracellular proteases and then moved by an ATP-dependent transporter mechanism to the lumen of the endoplasmic reticulum (Goldberg & Rock, 1992; Neefjes, Momburg, & Hammerling, 1993). In an as yet

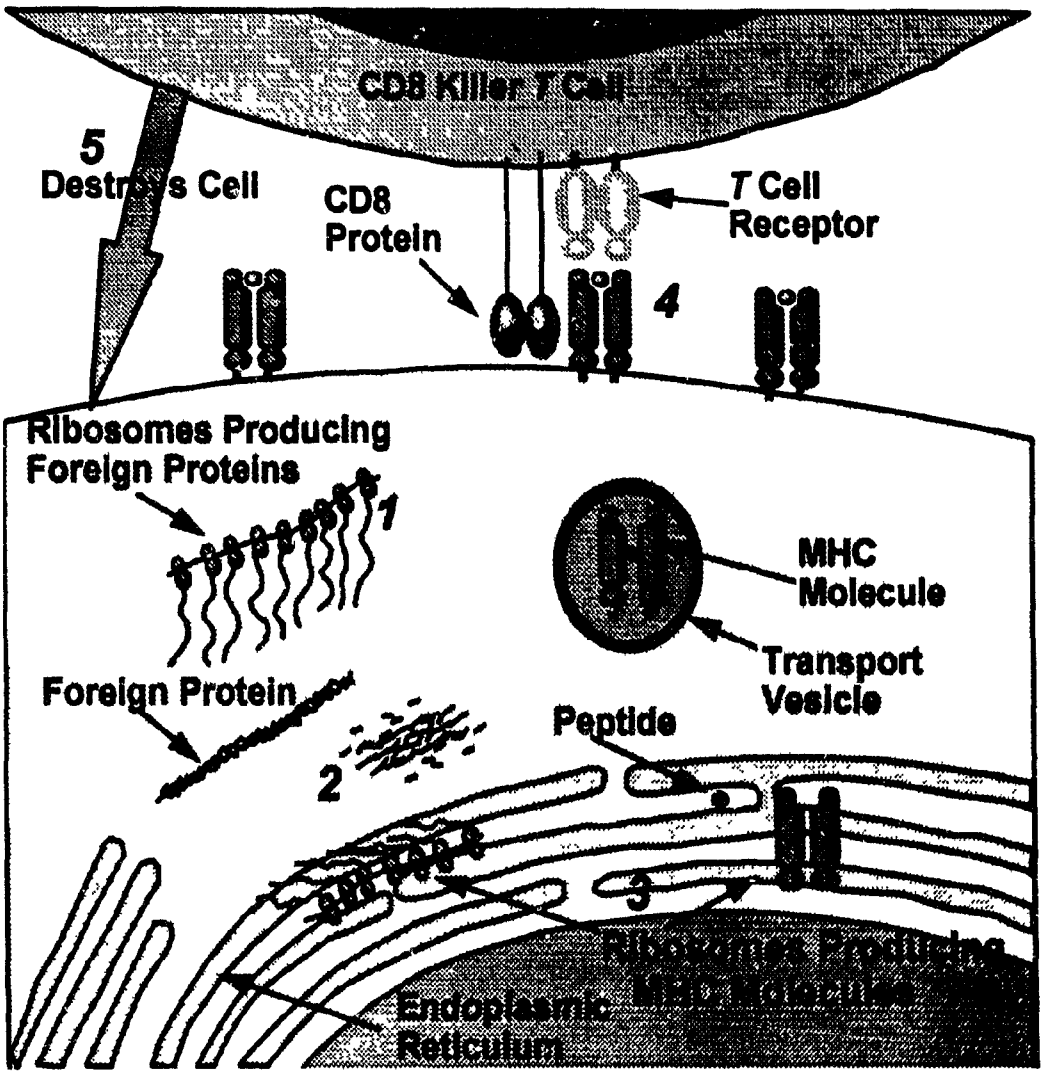


Figure 3.2. (Modified from Janeway, 1993) Viral proteins produced by an infected cell (*1*) are broken down into peptides (*2*). The peptides are taken to the endoplasmic reticulum, where class I MHC molecules form around them (*3*). Each complex goes to the cell surface. There it can be detected by a killer *T* Cell, which expresses a CD8 protein (*4*). The *T* cell then secretes compounds that destroy the infected cell (*5*).

undetermined manner, the MHC molecule is assembled around the peptide and transfers it to the cell surface where the immune response takes place if the peptide is foreign to the host organism (Townsend et al., 1989). The class Ia molecule also presents bacterial antigens which appear to have been processed within vesicular organelles rather than in the cytosol (Pfeifer et al., 1993). On the cell surface, the MHC-bound peptide is recognized by specific T lymphocytes (Zinkernagel & Doherty, 1974). Cytotoxic T lymphocytes with CD8 proteins on their surface bind with class Ia molecules (Parnes, 1989). The activated T lymphocyte binds with the complex at the 3 residue and secretes compounds which destroy the infected cell.

The majority of peptides which bind with the MHC molecule are nine residues in length (Madden, Gorga, Strominger, & Wiley, 1991); however, different peptides are bound by different class I molecules. The type of peptide which binds with the MHC molecule is determined by the structure of the binding cleft and its side pockets (Garrett, Saper, Bjorkman, Strominger & Wiley, 1989; Zhang, Young, Imarai, Nathenson, & Sacchettini, 1992). Two non-variant side pockets bind the amino and carboxyl terminals of all peptides (Silver, Guo, Strominger, & Wiley, 1992). The residues in the remaining pockets bind peptides selectively (Parker et al., 1992; Zhang, Gavoli, Klein, & Masucci, 1993). The β2-microglobulin chain stabilizes the peptide complex during folding and assembly (Saper, Bjorkman, & Wiley, 1991).

This peptide binding selectivity is a result of the presence of different amino acid residues in the binding cleft of different MHC molecules. Thus, the genetic diversity of the MHC contributes to the ability of an individual to bind a greater variety of antigenic peptides and present them to a T cell receptor. It should be noted, however, that because each individual has a limited number of different MHC molecules, each allele binds a wide variety of distinct peptides. Consequently, it is likely that one MHC molecule or another

will be able to bind a peptide from most pathogens (Engelhard, 1994) so that every individual can mount an efficient immune response to the majority of pathogens.

The ability of the MHC molecule to selectively bind molecules has generated a theory as to the origin of odours of individuality that are found in the urine of mice and rats. This "carrier hypothesis" suggests that the unique urine odour arises from volatiles which bind to the pocket of a degraded MHC glycoprotein and are released in the urine following excretion of the molecule (Roser, Brown, & Singh, 1991; Pearse-Pratt, Schellinck, Brown & Roser, 1992). The specificity of the volatiles would depend upon the differential binding sites in the cleft of the MHC molecule.

It has been estimated that a single MHC molecule can bind up to 2000 different peptides (Hunt et al., 1992, Engelhard et al., 1994). If each cell expresses 10^5 - 10^6 MHC molecules as suggested by Englehard (1994), the potential for peptide binding is enormous. If an equivalent number of odour molecules can also be bound in the MHC pockets, it does not seem likely that each individual would bind a unique pattern of volatiles. Nonetheless, until further hypotheses as to the way that MHC differences are expressed in the urine are proposed and investigated, this theory should not be dismissed.

With advances in the understanding of how MHC molecules are formed has come the ability to produce class Ia- and class II-deficient mice as well as double-deficient-class Ia and class II mice (Raulet, 1994; Cardell et al., 1994). At this time, this research is in the initial stages and while the mice are deficient in class I molecules, they still show a low level of functional class I molecules (Bix & Raulet, 1992). These mice have been primarily developed for the study of immune system processes and transplantation procedures. As totally class I deficient mice are engineered and as they become available for behavioural studies, the role of these particular MHC molecules in the production of individually unique odour phenotypes should become clear.

3.1.4. Class Ib genes

The class Ib genes are usually referred to as the "non-classical class I genes" because they do not appear to be involved in the presentation of antigen to cytotoxic T lymphocytes. Initial reports indicated that these genes were only expressed in hematopoietic cells (Chen et al., 1985; V nachio et al., 1989). Recently, a specific class Ib gene has been found to be widely transcribed in such tissues as the brain, heart, lung, kidney, liver, spleen and muscle (Palmer & Frelinger, 1987). Some of the Q region molecules are also expressed as secretory molecules in the serum (Soloski, Vernachio, Einhorn, & Lattimore, 1986).

The glycoprotein r.roducts of these genes are similar in structure to the class Ia antigens (Prochnicka-Chalufour, Casanova, Kourilsky, & Claverie, 1989). As their function is unclear, however, interest in the class Ib genes both from the perspective of the immunologist and the behaviourist has been limited (Srivastava, Ram, & Tyle, 1991; Brown & Egid, 1994). Some immunologists have, in fact, referred to the class Ib genes as an "evolutionary junkyard" (Klein & Figueroa, 1986); whereas others have speculated that they may be involved in the regulation of growth and development and maternal/fetal interactions (Flaherty, Elliott, Tine, Walsh, & Waters, 1990; Schmidt & Orr, 1993). Stroynowski and Fischer Lindahl (1994) have reviewed the mounting evidence suggesting that these proteins can bind peptides and are involved in antigen presentation in a manner similar to that of the class Ia molecule.

In what way could these class Ib genes contribute to signals of individuality? The polymorphism which is so characteristic of the class Ia genes is much less prevalent in the class Ib gene regions (Stroynowski, 1990) although there have been reports of multiple alleles in the genes of the Q region (Watts et al., 1989; Stroynowski & Fischer Lindahl, 1994). Regardless of the level of polymorphism expressed in any single gene, there are many more genes found in this portion of the MHC than in the classical MHC region.

For example, in the Qa and Tla regions of the mouse, at least 48 genes have been isolated (Smith, Steinmetz & Hood, 1987). Moreover, another cluster of genes termed the Hmt region has been linked to the Tla (Chorney, Mashimo, Chorney, & Vasavada, 1991). These genes function in the transmission of maternal antigen (Fischer Lindahl, Hausmann, & Chapman, 1983) and it is not known if they are involved in phenotypic variation. Nonetheless, our knowledge about class Ib genes is constantly being updated and it may indeed be the case that they are the "hidden treasure of the MHC junkyard" (Flaherty et al., 1990).

In contrast with the consistent expression of class Ia genes, the number of class Ib genes expressed in different strains of mice varies (Chorney et al., 1991). It is possible that the variation in the number of functional genes and differences in the expression of their products could influence characteristics of an individual in a manner which could be communicated to a conspecific (Flaherty et al.,1990). The *Ped* gene (Q9), which is encoded on the Qa-2 region of the H-2, is one gene that could function in this manner. This gene modulates the development of preimplantation embryos. Consequently, mice with a certain *Ped* allele (i.e., the *Ped* fast allele) will have larger litters and larger pups than those with the *Ped* slow allele (Warner, Brownell, & Rothschild, 1991; Xu, Jin, Mellor, & Warner, 1994).

The *Ped* gene had not been identified at the time of the experiments of Andrews and Boyse (1978) and Yamazaki et al.(1982). Nonetheless, it may be significant that two strains of male mice given a choice between females which differ at the Qa-2 region choose to mate with females encoding the *Ped* fast gene (Andrews & Boyse, 1978). Similarly, the odours of mice differing in this region could be discriminated in a Y-maze paradigm (Yamazaki et al., 1982). At the time of testing, the mice in both of these studies also differed in the genes of the class Ib Tla region; consequently, one cannot make a definitive

conclusion regarding the discriminability of products of the *Ped* gene. Regardless, the fact that the odours of strains which differ in the non-classical MHC genes are discriminable suggests that the possible role of the non-classical MHC genes in the production of unique phenotypes merits further investigation.

3.1.5. Class II genes

The expression of class II MHC molecules is limited to cells of the immune system such as T and B lymphocytes and macrophages. They bind peptides derived from exogenous proteins which have entered the cell by endocytosis (Allen, 1987). The class II molecule then presents the peptide to helper T-cells or inflammatory T-cells which express the class II binding protein CD4 (Parnes, 1989). The inflammatory T-cell then stimulates the cell to destroy phagocytosed foreign peptides; helper T-cells activate B lymphocytes to secrete antibodies against the presented peptide (Janeway, 1993).

While it likely that the class I and class II molecules are derived from the same ancestral MHC molecule, their structures are somewhat different (Lawlor, Zemmour, Ennis, & Parham, 1990). Although the class II molecule also consists of a four domain extracellular structure, both the alpha and beta chains are variable and are encoded in the MHC. Consequently, it is possible that variation in the class II molecule could result from different subunits forming a functional molecule (Charron, Lotteau, & Turmel, 1984; Lotteau, Teyton, Burroughs, & Charron, 1987). As the peptide binding groove is comprised of one alpha and one beta chain, this variation could result in different peptide binding specificities.

The peptide binding site of the class II molecule is open at both ends (Brown et al., 1993). Consequently, the peptides which bind to the class II molecule are longer and of more variable length than those associated with class I molecules (Engelhard, 1994). This

difference combined with the existence of multiple isotypes and the possibility of interisotype pairing provide the class II molecule with further sources of structural diversity which may enable it to bind many different peptides.

3.1.6. Class III genes

Four class III genes have been mapped within the MHC (Carroll, Campbell. Bentley & Porter, 1984). These genes code for serum proteins and cell surface receptors of the complement system. HLA typing has revealed that these genes are likely polymorphic (Olaisen, Teisberg, Gedde-Dahl, Thorsby, & Jonassen, 1983). Variability of gene organization in Class III regions among individuals has also been reported but it is not known if these variations produce any functional modifications (Chimini & Pontarotti, 1991).

Although the complement encoding genes may not be involved in the production of unique phenotypes, other genes which map to the class III region may be influential in this regard. The pair of tumor necrosis factor (TNF) genes produce cytokines which are cytotoxic to tumor cells and other transformed cells (Collins, Lapierre, Fiers, Strominger, & Pober, 1986). TNF in human cells also acts to amplify the transcription of class Ia genes (Collins et al., 1986). As the surface expression of antigens is increased, it follows that products which reflect individuality could also be enhanced.

The heat shock proteins, generally noted for their role in the regeneration of stress-damaged proteins (Lindquist, 1988) also appear to be involved with the association of antigen with MHC class II molecules (DeNagel & Pierce, 1993). In addition, the genes controlling susceptibility to orchitis and cleft palate are within the so-called Hsp70-Bat-5 class III region (Snoek et al., 1993). It appears that, until the mechanism by which cues of

individuality are presented is more precisely defined, we cannot dismiss the potential role of any genes within the MHC complex.

3.2. Genes linked to the MHC

Prevailing theories associate the origin of discriminable phenotypes with the products of the Class Ia and Class II MHC genes (Brown et al., 1989; Boyse, Beauchamp, Yamazaki, & Bard, 1991). It is, however, possible that other genes which are closely linked to the MHC loci are also relevant in this regard. In wild populations, individuals will likely vary at these regions as well at MHC loci. Moreover, depending upon the method of derivation of congenic strains, the differential segment contributed by the donor strain of laboratory animals may be much greater than expected (Klein, Tewarson, Figueroa, & Klein, 1982). Thus, some of the effects attributed to specific MHC loci may be caused by unidentified linked loci. Consequently, the possible effects of such loci in creating discriminable phenotypes should be carefully evaluated.

One such genetic region which should be considered in this regard is the growth and reproduction complex (*grc*) of the rat. The genes of this region segregate with the class I RT1.E locus (Gill & Kunz, 1979). Among the characteristics associated with homozygozity at the*grc* are decreased growth, decreased female fertility, male sterility, small testes, and postnatal death of offspring (Kunz, Gill, Dixon, Taylor, & Greiner, 1980). In humans, there is increasing evidence that a similar MHC linked gene is at least partially responsible for reproductive failure (Gill, 1992).

There is also an indication that this complex affects susceptibility to cancer causing agents. Rats fed a diet containing a chemical carcinogen developed metabolic abnormalities associated with the development of cancer if they were homozygous for the *grc* (Rao,

Shinozuka, Kunz, & Gill, 1984). It seems plausible that the presence or absence of some of these characteristics coul ` influence the physiology and metabolism of individuals in a manner that could be perceived by conspecifics. The endocrine profile of these individuals is similar to that of normal rats (Greiner, Gill, Kunz, & Gay, 1980) so it would not appear that differences in levels of FSH, LH or testosterone contribute to any discriminable differences in the odours of these rats.

The MHC has also been linked with several aspects of corticosteroid physiology. For example, the enzyme 21-hydroxylase which is involved in steroid synthesis in the adrenal cortex is encoded on a gene within the class III region (White, New, & Dupont, 1986). H-2 linked genes also influence the level of expression of glucocorticoid receptors involved in anti-inflammatory responses (Goldman & Katsuma, 1986). In addition, H-2 congenic strains differ in levels of an isomerase which participates in the catabolism of corticosteroids in the liver (Monder, Walker, & Bradlow, 1982). As corticosteroid metabolites are ultimately excreted in the urine (Bradlow & Monder, 1982), it is possible that they may influence the discriminability of urine odours. While the discriminability of corticosteroid metabolites has not been tested, it has been established that steroid metabolites do have distinct odours and there have been speculations that they may function as sex attractants or human pheromones (Kloek, 1961). Thus, it seems evident that the MHC may influence odours indirectly through its effect on metabolism as well as through the action of its class I and II molecules.

Ivanyi (1978) compiled a list of physiological and pathological traits in which MHC congenic mice may differ and suggested that the H-2 complex influences hormone metabolism. He documented differences in blood testosterone and testosterone binding globulin and cytosolic cortisol-binding globulin protein levels. The mass of the vesicular gland, the testes and the thymus also showed quantitative variations correlated with MHC-

type. At the time this research was undertaken, the sophisticated techniques necessary to determine what loci were associated with particular traits were unavailable. Consequently, it is impossible to know if the H-2 system or linked genes are responsible for such influences. Regardless, it seems likely that variation in such characteristics could be expressed phenotypically.

3.3. Summary

The MHC plays a critical role in the immune system response by encoding for molecules which present small peptide fragments of antigens on the cell surface for subsequent processing by T or B lymphocytes. Because of the polymorphism of the classical class I and class II MHC loci, it has been postulated that they are responsible for conferring upon rodents a unique odour phenotype which can be discriminated by conspecifics. The structure of the MHC binding cleft may provide the mechanism by which unique volatiles are transported to the urine. As non-classical class I genes such as the *ped* gene in the mouse may influence embryonic development and reproductive performance, they may also contribute to phenotypic individuality. In addition, genes which affect susceptibility to disease and are also related to growth and development, such as the *grc* in the rat are tightly linked to the MHC. The role of these non-classical MHC genes in the production of discriminable odours of individuality has yet to be thoroughly investigated. Until proven otherwise, their potential should not be dismissed.

CHAPTER 4

BEHAVIOURAL EVIDENCE FOR THE ROLE OF THE MHC IN PRODUCING ODOURS OF INDIVIDUALITY

The task of determining which genetic region might influence the production of odours of individuality was made easier by a serendipitous observation at the Sloan Kettering Memorial Cancer Centre, New York. Technical staff noted that homozygous males of an inbred strain of mice (AKR-H-2^b) housed with heterozygous (AKR-H-2^b:H-2^k) and homozygous females (AKR-H-2^b) appeared to show a mating preference for the heterozygous females (unpublished data, reported by Yamazaki et al.,1976). As these female mice differed genetically only at the major histocompatibility complex, it was hypothesized that the MHC could be involved in the production of odours of individuality. Subsequently, both mate choice tests and operant conditioning experiments were undertaken over the next twenty years to assess the role of the MHC in this context. The results of some of these experiments will be critically examined in this chapter.

4.1. Mate Choice Experiments in Male Mice

To verify the mate choice observations, Yamazaki et al., (1976) designed an experiment to determine if inbred male mice of different strains showed a consistent strain preference for females of a different MHC haplotype. In these initial experiments, the mice differed in the entire chromosomal region which included the H-2 complex rather than differing in alleles of a specific H-2 gene. The outcome of this experiment has provided the "raison d'etre" for all of the subsequent behavioural research regarding the role of the MHC

in creating discriminable odours. Consequently, it is worthwhile to carefully examine the results and their subsequent analyses.

A series of tests (6 tests per mouse) were conducted in the home cage of each male subject into which a pair of H-2 congenic estrous females were placed. The mating preferences of homozygous males from several different pairs of congenic strains of mice were assessed with the following female pairs: (1) a genetically identical homozygous female and a dissimilar homozygous female; (2) a genetically identical homozygous female and a heterozygous female with whom he shared at least one H-2 haplotype; (3) a dissimilar homozygous female and a heterozygous female with whom he shared at least one H-2 haplotype. The preferences of heterozygous males derived from two different pairs of MHC congenic mice were also determined.

The investigators reported that homozygous males from four of the six strains showed a preference for females with an H-2 type "dissimilar" from themselves, one strain showed a preference for females of "similar" strains and one strain did not show a preference. Of the two strains of heterozygous males tested, one strain showed a preference for "dissimilar" females and the other showed no preference.

There is one problem with these experiments which may have masked the preferences for females of a specific MHC type. The same test males (e.g., "kk") were required to choose between hybrid and homozygous strains (e.g., between "kk" and "bk" or between "bb" and "kb") as well as between homozygotes (e.g. "kk" and "bb"). If a male (e.g., "kk") preferred to mate with the most dissimilar H-2 type, he would be required to choose a bk female over a kk female on one trial but on a subsequent trial, in which a bk female was presented with a bb female, he would be required to choose a bb female and reject the bk female. Consequently, it is possible that a clearer picture of preference would be manifest if each male was presented with only one type of trial.

Although Yamazaki et al. (1976) used the total number of trials as the N in all calculations, this may have been inappropriate in a repeated measures design (Kirk, 1968). By using a larger N, the 95 % confidence interval is shortened and a significant result is more likely to occur. Using the data provided in the publication, I re-analysed the data using the number of males of each homozygous strain tested as the N. I calculated the 95 % confidence interval, estimating the standard error of proportions as recommended by Ferguson (1981).

The results of my more conservative analysis indicated that three out of six strains tested did not show a preference. This contrasts with the interpretation of Yamazaki et al. (1976) who found that only one strain did not show a preference. While this reanalysis detracts somewhat from the original findings, it does not negate the fact that, in some instances, the males of specific strains of mice were able to use cues provided by the H-2 complex to discriminate between congenic females.

Inbred strains of mice maintained over many generations may acquire characteristics unrelated to their specific genetic differences. To exclude the possiblity that genetic drift or residual genotypic differences were responsible for mate choice, the mating preferences of homozygous F_2 segregants were investigated (Yamazaki, Yamaguchi, Andrews, Peake and Boyse, 1978; Andrews & Boyse, 1978; Yamaguchi, Yamazaki & Boyse, 1978). The results were equivocal (i.e., some results were significant regardless of which N used), with males of some strains showing preferences for dissimilar homozygous females and others showing no preference.

Interestingly enough, the authors suggested that by testing F_2 segregants, it would be possible to control for possible differences in commensal bacteria which were unrelated to MHC haplotypes but which might produce discriminable characteristics. At this same time, Howard (1977) was hypothesizing that the types of bacteria present in the gut were actually

controlled by the MHC genes and could be responsible for the mating preferences found by Yamazaki et al., (1976).

Yamazaki et al. (1976) suggested that two types of recognition genes were expressed by the MHC, a gene which coded for the identity of the signaling individual and a response gene that determined how an individual would respond to the presented signal. If the MHC could be shown to encode such a genetic recognition system, this finding would substantiate Hamilton's (1964) theory that kin recognition functions by the genetic coding of both cue production and response mechanisms.

While some results were compatible with this theory (Andrews & Boyse, 1978; Yamaguchi, Yamazaki, & Boyse, 1978), it should be noted that in all of the mate choice studies so far described, the males were congenic or syngenic with the pair of test females. Consequently, it is possible that those males which did exhibit significant choice behaviour may have done so by discriminating on the basis of familiarity. Such an hypothesis does not imply that specific cues produced by the MHC could not form the genetic basis of individuality. Rather, it suggests that no matter how the unique characteristics of an individual are determined, the differential cues which result may be learned as a consequence of associations between conspecifics.

Yamazaki et al. (1988) examined the role of familiarity in male mate choice by comparing the mating preferences of mice fostered to a genetically identical individual with the preferences of those cross-fostered to an individual which differed genetically from themselves only at one particular MHC locus. They found that the males preferred to mate with a female of the different strain when they were in-fostered by parents of their own strain but they reversed their preference and preferred females of their own strain when they were cross-fostered to parents of another strain. Results such as these confirm the ability of male mice to discriminate based upon MHC cues and emphasize the importance of

early experience in learning the cues on which mate preferences are based. They also substantiate earlier reports which describe the influence of rearing odours on sexual responsiveness in rats (Marr & Gardner, 1965) and mate choice in mice (Yanai & McClearn, 1972a-c).

Two specific criticisms of the studies conducted by Yamazaki and his colleagues were addressed by Eklund, Egid and Brown (1992). Rather than using laboratory mice bred to be MHC congenic, they examined the mating preference of laboratory mice which had naturally occurring MHC haplotypes isolated from wild mice. To eliminate the possiblity that interactions between the two unrestrained female mice might influence the male's choice during the test trials, they used a three chambered preference apparatus rather than the male's home cage. The male subject was required to choose one of two tethered females located in the side chambers. Under these conditions, neither of the two strains of mice tested showed any preference for females of the two strains. It is not possible to determine what factors may have contributed to this lack of support for the findings of Yamazaki, Boyse et al. (1976) and Yamazaki, Beauchamp et al. (1988). Definitive conclusions as to the role of the MHC in male mate choice await the adoption of a consistent and appropriate methodology and the testing of many more strains of MHC-typed mice.

At the time of Yamazaki's initial research, sophisticated procedures for mapping chromosomal regions were unavailable. Consequently, it is impossible to say how the different MHC genes or non-MHC chromosomal regions included within the H-2 complex may have influenced the mate choice of the males. Currently, techniques such as gene amplification by polymerase chain reaction and pulse field gradient electrophoresis can determine the precise character of the MHC haplotype (Dyer et al., 1993; Srivastava, Ram & Tyle, 1991). As well, MHC congenic mice which differ only at specific loci can be

produced. To further advance our knowledge of the role of the MHC in mate choice, it would seem appropriate to use such derived strains of mice to repeat the original mate choice experiments. This approach may not be without its pitfalls, however. In attempting to assess the influence of particular genetic loci on behaviour, one must guard against producing inbred strains of mice whose characteristics do not reflect those of a natural population.

4.2. Mate choice experiments in female mice

As the females of a species are generally considered to have a greater parental investment than males (Bateson, 1983), it follows that they would be expected to exhibit significant mate preference behaviour. An examination of the experimental evidence implicating the MHC in determining female mate choice reveals that females do, in some instances, choose to mate with specific strains of males (See Manning, Potts, Wakeland, & Dewsbury, 1992 for a review). While there are some inconsistencies in the research findings, it appears that the female mate choice data are less contradictory than that of the males discussed in section 4.1. The mating preference studies described used several different paradigms in which the type of access to males, the haplotype of the females and the housing conditions were manipulated.

As a corollary to their study on the role of preweaning experience in mate choice in male mice, Beauchamp et al. (1988) found that cross-fostering of females by congenic parents did not influence their subsequent mating preference. Females of both strains (H-2^b & H-2^k on a B6 background) preferred the same strain (H-2^b). Partial confirmation was found for these results when the preference of males of the same congenic strains tethered in a test arena rather than presented unrestrained in the female's home cage was

examined (Stahlbaum, 1988). H-2^b females showed a preference for their own strain but H-2^k females did not.show a preference As females of neither strain showed a preference for H-2^b males on an AKR background, Stahlbaum (1988) suggested that B6 (H-2^b) females may base their choice upon both the H-2 similarity and a possible interaction of H-2 and background genotypes.

Using an apparatus similar to that of Stahlbaum (1988), Egid and Brown (1989) investigated both the odour preference and mate choice of MHC congenic females for males with a laboratory established genetic background and MHC haplotypes isolated from wild mice. Estrous females of both strains preferred the bedding odours of MHC dissimilar males but metestrous females showed no preference. In this study, choice was determined as the first male to ejaculate and females from both strains also chose to mate with an MHC dissimilar male. The positive findings of the earlier female mate choice studies are strengthened by results such as these in which mice with MHC haplotypes derived from wild mice were tested. Using the data from this study and that of Potts, Manning and Wakeland (1991), Hedrick (1992) developed a model of female mate choice. He determined that the degree of preference shown in these studies is sufficient to maintain MHC polymorphism.

Potts, Manning and Wakeland (1991, 1992) examined the mating patterns and MHC type of offspring of half-wild female mice with specifically derived MHC haplotypes maintained in outdoor enclosures. They calculated there to be a mean deficiency of 27 % homozygous offspring over all of the populations and they concluded that this would be significantly fewer homozygotes than would be expected from random mating. Their analysis revealed that outbreeding primarily occurred because females left their home territory and mated with MHC dissimilar males from other areas; however, an excess of territorial matings with heterozygous males also contributed to this difference.

It appears to me that the calculation to determine a homozygote deficiency in six out of nine instances is not significantly different than that expected by chance [See Table 2, Potts et al., (1991)] . As the populations are not identical in genetic background, the percent of homozygosity in each group should not be combined into one mean to assess significance. Thus, while these results are suggestive of a role for the MHC in determining female mate choice in an ecologically valid system (i.e., three populations with a significant homozygote deficiency), further work is needed to confirm this conclusion.

The most convincing evidence for the role of the MHC in the production of individual odours comes not from the mate choice studies but from a study of communal nesting patterns in a seminatural population of house mice (Manning, Wakeland & Potts, 1992). An analysis of the degree of MHC similarity between females in the chosen nest compared with the mean MHC similarity of all other nests available revealed that individual females chose to cohabit with females of more similar MHC types. Whether the choice of such an MHC similar female as a nestmate is evidence of kin recognition has been questioned (Grafen, 1992). Regardless of the purpose of these communal nesting activities, these results indicate that the discriminability of MHC-related odours does influence the behaviour of female mice. Moreover, as Brown & Eklund (1994) point out, this study has provided the first demonstration that the MHC can be used in the context of establishing a cooperative relationship among genetically similar individuals.

4.3. Discrimination of MHC congenic males in the context of pregnancy block

Pregnant female mice who are exposed to an unfamiliar male or his odour will experience termination of their pregnancy, i.e., pregnancy block. By mating females with males of one MHC haplotype and then exposing them to MHC congenic or syngenic males

or the urine of these individuals, Yamazaki et al., (1983) demonstrated that a difference at the MHC was sufficient to initiate pregnancy block. Further investigation revealed that pregnancy was blocked by a male which differed from the stud male only at a specific Class 1 gene (H-2K), rather than throughout the MHC (Yamazaki et al., 1986).

In these studies, the incidence of pregnancy block in females exposed to novel males was much lower (e.g. 36 - 58 %) than that reported by other laboratories (e.g. 83 %: Dominic, 1965; 88%: Kaba & Keverne, 1988). In the other experiments, the novel and stud male were not of such closely related strains and would likely differ throughout the MHC as well as in the rest of the genome. Thus, the lower rate of pregnancy block may be a result of the similarity of the odour profiles of the two males.

Also, in contrast to other reported findings (Brown, 1985), the incidence of pregnancy block to the stud male was high (e.g. 11 - 20 %) and the presence of a familiar female (19 %) or novel female (40 %) was found to mediate pregnancy block. It is not obvious what aspects of the experimental procedure could have contributed to these differences although stress of the females due to handling has been reported to increase pregnancy block in mice (Brown, 1985).

4.4. Discrimination of MHC variants in a Y-maze

While the results of the mate choice experiments, in particular those involving preferences of males, may be ambiguous in confirming the role of the MHC in producing discriminable odours, the studies in which mice are trained to discriminate between the odours of MHC congenic mice for water reinforcement in a Y-maze are more consistent. In a series of operant conditioning experiments, Yamazaki and his co-workers confirmed the ability of mice, trained in a Y-maze, to discriminate between the odours produced by

MHC congenic strains of mice. First, they showed that C57BL/6:H-2^b (B6) and C57BL/6:H-2^k (B6-H-2^k) congenic mice could be trained to discriminate between males of the same two congenic strains which were housed in containers at the end of each runway (Yamazaki et al., 1979). Further testing with these same subjects indicated that F_2 homozygotes could also be discriminated.

In a complementary study, Yamaguchi et al. (1981), using the same test subjects again, found that the urine odours alone from individual, same sex, MHC congenic donors provided discriminable cues. Usually urine from only one animal made up each stimulus; different donors provided urine odours for each trial. A control study was included in this experiment such that the test mice were required to discriminate between different individuals of the B6 strain, i.e., genetically identical animals. The urines from the B6 animals with the same MHC type were found to be indiscriminable.

It should be noted that the subjects were given significantly fewer trials to discriminate B6 animals of the same MHC type than they received in the discrimination training between the urine odours from the MHC congenic mice. For example, one test mouse received approximately 1100 trials with congenic urines but only 240 trials with urine fron two mice of the same strain. In addition, the same mouse had previously been trained with the same strains of MHC congenic mice as odour sources (1481 trials). Thus, this particular experimental animal had nearly eleven times as many trials to learn the between strain discrimination as it had to learn the within strain discrimination. Similar discrepancies in the amount of training were found for the other subjects.

These studies established the finding that mice are able to discriminate between the urine odours or body odours of other mice which differ genetically only at the MHC. However, because of the lack of adequate control trials, the possibility that genetically identical individuals may be discriminated should not be dismissed. Rats are, in fact, able

to learn such a discrimination. In an examination of the ability of rats to discriminate between the urine odours of conspecifics, Schellinck, Brown and Slotnick (1991) found that the odours of both MHC congenic rats and genetically identical animals could be discriminated. Differences between odours from MHC congenics were, however, learned more quickly and remembered from session to session. It appears that non-MHC factors may contribute to the individual differences between odour donors, a hypothesis which can only be tested if genetic cues are kept constant and non-genetic cues such as diet and hormonal levels are manipulated.

As genetic recombinant techniques became more sophisticated, mice could be produced that differed in very specific genetic regions. Consequently, Yamazaki et al. (1982) were able to investigate the role of different regions of the H-2 complex in the production of individual odours. By training mice in a Y-maze, they showed that genetic differences limited to the Qa:Tla region as well as to the K region of the H-2 could produce discriminable urine odours (See Figure 3.1). Using an identical procedure Yamazaki et al. (1983) determined that urine odours from individuals which have different alleles of the H-2^k gene could be discriminated.

In a further demonstration of the discriminability of minor genetic differences at the MHC, Yamazaki, Beauchamp, Bard and Boyse (1990) showed that a number of other single gene mutations of the H-2^k gene produced recognizable differences in urine odours. In this same study, the urine odours of mice which differed only at the class II region of the MHC were also discriminated, an indication that a mutation of these genes is also sufficient to create an individual odour. Yamazaki et al. (1990) found that not all mice with mutant forms of H-2^k were discriminable. As it is not known how differences in MHC glycoproteins are translated into discriminable odours, one cannot determine why some mutants are discriminable and some are not.

Yamazaki, Beauchamp, Shen, Bard and Boyse (1994) reported that the odours of mice with known MHC types but varied backgrounds could be readily discriminated by mice trained in a Y-maze and concluded that differences in the background genes did not disrupt the ability of mice to use cues based only upon constant H-2 differences. Such a result demonstrates very clearly the relevance of the MHC as a genetic marker for odours of individuality. Whether cues generated by genetic differences are more relevant than information regarding an individual's diet or the status of its health has yet to be determined.

4.5. Discrimination of other genomic variants of mice

The studies discussed in section 4.4 support the position that odours of individuality originate within the MHC; however, they do not rule out the possibility that other genetic differences could also influence the production of individual odours. Yamazaki et al. (1986) considered the role of X and Y chromosomes in this context. Using the Y-maze, they showed that urine from mice of two strains, genetically identical except for differences in the X and Y chromosomes, could be discriminated. The urines were discriminable whether the mice differed in both the X and Y chromosomes or only the X or Y chromosome. The urines of females with identical X chromosomes could not be discriminated. Further evidence for the powerful effect of differences in Y chromosomal odours was demonstrated by the report that urine from Y chromosome congenic mice caused pregnancy block (Yamazaki, Beauchamp, Bard, & Boyse, 1989).

The Y chromosomes of the two mouse strains used by Yamazaki et al., (1986) were derived from two different species of mice, *Mus musculus* and *Mus domesticus* (Nishioka, 1987). Monahan, Yamazaki, Beauchamp and Maxson (1993) used the Y-maze

paradigm to verify that urine odours from two Y chromosomal variants derived from the same species, *Mus musculus*, were also discriminable.

Genes on the Y chromosome influence serum levels of testosterone (Novotny, Harvey, & Jemiolo, 1990). To determine if testosterone mediated cues were responsible for the discriminable cues found in the urine of Y chromosomal variants, Schellinck, Monahan, Maxson and Brown (1993) compared the ability of rats trained in an olfactometer to discriminate between urine odours of intact or castrated Y congenic mice. The urine odours from gonadectomized Y congenic males were discriminated as readily as the urine odours from intact Y chromosomal congenics. Moreover, neither of these tasks were more difficult than a discrimination of urine from MHC congenic male mice.

It is not known which genes on the Y chromosome contribute to discriminable odour cues. As the Y chromosome is not a part of the genetic makeup of females, it is possible that it contains information denoting the sex of an individual rather than differences between individuals. However, it should be noted that two genes on the Y chromosome code for minor histocompatibility antigens (Mclaren, 1987; Muller & Latterman, 1988), one of which may have strain variants (Scott et al., 1991). The weight and morphology of kidneys and the weight of adrenal glands are also influenced by genetic variability in the Y chromosome (van Abeelen, Leenders, & Lemmens, 1990). As MHC-linked genes also influence corticosteroid metabolism (White, New & Dupont, 1986) and bacteria are involved in the production of steroid end-products (Macfarlane & Cummings, 1991), it seems that a potential role for corticosteroid metabolites in contributing to odours of individuality should not be dismissed.

In a report of previously unpublished data, Yamazaki, Beauchamp, Bard and Boyse (1990) compared the number of training trials to reach criterion by mice trained to discriminate between the urine odours of MHC congenic mice and the number of trials to

criterion for mice trained to discriminate between the odours of mice which differed only at the X or Y chromosome. Although a statistical analysis was not performed, the mean number of trials to criterion was greater for differences at the Y chromosome and X chromosome than for differences at the MHC. Yamazaki et al., (1990) suggested that these data are indicative of the greater saliency of differences produced by the MHC than by the sex chromosomes. In making these observations, the authors were comparing data collected in different experiments and the degree of pretraining may have varied. It would be helpful to examine the performance of the same subjects on the different types of odours within the same experiment prior to coming to conclusions regarding the relative contribution of various genetic regions to an odour profile.

There have been several reports that differences in other non-MHC regions of the mouse genome also provide discriminable odour cues (Beauchamp, Yamazaki, Duncan, Bard, & Boyse, 1990; Boyse, Beauchamp, Bard, & Yamazaki, 1991; Eggert, Holler, Luszyk, & Ferstl, in press). The presence of a discriminable difference in the urine of individuals differing in undefined genetic regions (or in the X or Y chromosomes) does not confirm a role for these genetic regions in individual recognition. Operant conditioning paradigms, in which the subject's level of motivation has been manipulated by food or water deprivation, have generally been used in these experiments. A demonstration that these differences are used to discriminate between individuals in the context of mate choice such as has been shown for the MHC in relationship to female mate choice (Egid & Brown, 1989) or communal nesting patterns (Manning et al., 1992) would be useful in confirming the significance of these odour differences.

4.6. The expression of MHC haplotypes in mice

The studies described above have adequately demonstrated that differences between MHC congenic strains of mice or their urine odours are discriminable; however, the way in which the differences at the MHC are translated into distinctive odours is not known. As discussed in Chapter 3, the odours may be bound to the MHC molecule or the MHC may modulate the production of volatile steroid metabolites. Yamazaki and his colleagues have tested a number of hypotheses to determine how this difference might be expressed.

As the discriminable odours are present in the urine, it is possible that the MHC-related odours may be produced by the kidney. To determine whether the kidney or the hematopoietic system was the source of the odourants, Yamazaki, Beauchamp, Thomas and Boyse (1985) trained mice to discriminate between the urine odours of B6 and B6:H-2^k males. These subjects were then given non-reinforced transfer of training trials in which the urines to be discriminated were from radiation chimeras composed of irradiated B6 individuals injected with bone marrow from either a B6 or B6:H-2^k mouse. The mice responded to the urine odours as if they were from the donor animals, i.e., from a B6 or a B6:H-2^k mouse rather than from the two irradiated B6 mice. Thus, it seems likely that blood-forming cells take part in the production of odours of individuality.

An examination of the changes produced in the urine odour of fully allogenic mice, i.e., mice which differ in the MHC and background genes, produced inconsistent results (Eggert, Luszyk, Ferstl, & Muller-Ruchholtz, 1989). For example, mice rewarded for choosing the urine odour of a C3H mouse (S+ odour) transferred this training to respond to the odours of C57 mice which received the bone marrow of a C3H mouse. However, the mice still responded when the bone marrow of a C57 mouse was put into the bone marrow of a C3H mouse. This appears to indicate the mice were responding to both the

C57 and C3H odour as an S+. It is possible that both donor-specific and recipient -specific components play a role in determining odour constituion of fully allogenic chimeras. Without control trials from totally unknown donors, it is difficult to assess this hypothesis.

Following the report that bacteria play a role in producing discriminable odours in rats (See Section 4.7.2 for details), Yamazaki et al. (1990) showed that mice trained in a Y-maze were able to discriminate between the odours of mice which had been raised in germfree conditions. These findings may indicate that the odours of individuality are controlled by different mechanisms in mice and rats. Such a conclusion awaits further research in which the discriminablity of germfree samples from both species is assessed under the same experimental conditions.

Knowledge as to when odours of individuality develop could be helpful in determining the origin of their expression. MHC congenic mice housed in odour boxes in the arms of a Y-maze cannot be discriminated until they are 14 days old; however, the urine odours of 1-day-old mice can be readily discriminated (Yamazaki, Beauchamp, Imai, Bard & Boyse, 1992). As young rats only appear to urinate when stimulated by a mother's anogenital licking (Gubernick & Alberts, 1985), Yamazaki et al. (1992) suggested that it is likely the absence of urine which makes the actual mouse pups indiscriminable.

If a discriminable odour is present in a 1-day-old mouse, it is possible that the developing fetus also expresses an odour. In such an instance, the odour of a fetus could be transferred to the mother and subsequently alter the nature of her own odour (Yamazaki et al. (1992). To test this hypothesis, Beauchamp et al. (1994), collected urine from genetically identical females of different inbred strains (e.g. B6) which were pregnant with fetuses bearing both the maternal (e.g. B6) and paternal (e.g. B6-H-2k) haplotypes. Urine collected from these pregnant females when the fetuses were 13-18 days old was discriminable in the standard Y-maze task. This ability to discriminate such odours

generalized to previously undiscriminated test samples containing the different paternal haplotypes of 9-12 and 13-18-day old embryos but not to samples containing the haplotypes of the 6-8-day old embryos.

Beauchamp et al. (1994) interpreted these results to mean that the MHC class I gene products from the genetically dissimilar fetus had modified the odour of its mother. The nature of the maternal-fetal interaction is not well understood; in particular, the relationship of the maternal immune response to fetal antigens is unclear (Billington, 1993). It may be that the odour cue in the urine of a pregnant mouse is a product of the mother's physiological response to the presence of fetal antigens. Different fetal haplotypes could initiate different maternal immune responses which could result in discriminable maternal odour profiles. This possibility must be eliminated before one can conclude that a fetal haplotype directly changes the urine odour of its mother.

4.7. Urine as a carrier of MHC-produced signals

While the origin of the odours of individuality in mice is still in question, the results of the Y-maze experiments leave no doubt that urine is a carrier of these signals. The component of the urine which contains the individually unique odour remains elusive. Singer, Tsuchiya, Wellington, Beauchamp and Yamazaki (1993) examined the ability of mice to discriminate between urine samples which had been collected from MHC congenic strains of mice and subjected to a number of different fractionating procedures. Samples containing only urinary proteins could not be discriminated. Dialysis or ultrafiltration revealed that, after proteins were removed, components with a molecular weight of less than 3500-3000 daltons contained discriminable cues. Singer et al. (1993) concluded that proteins are unlikely to be directly involved with the expression of an individual's odour

profile. However, these findings do not eliminate the hypothesis that proteins may selectively bind metabolites present in the blood serum and release them in the urine where they act as discriminable odours (Singh, Brown, & Roser, 1987).

Tsuchiya, Yamazaki, Beauchamp and Singer (1992) reported that mice can discriminate between several fractions of urine separated by sequential high performance liquid chromatography. They did not indicate the chemical nature of these compounds but suggested that urine contains multiple redundant, simple olfactory cues which signal MHC type. In contrast, a GC-MS analysis of the urines from prepubertal MHC congenic female mice isolated several qualitative differences in the components present but found mainly quantitative differences in the pattern of secondary metabolites (Schwende, Jorgenson, & Novotny, 1984). No attempt was made to determine if mice could discriminate between any of these isolated mixtures.

Mouse urine is chemically very complex, with at least 60 volatiles being identified in samples from males and females (Schwende, Wiesler, Jorgenson, Carmack, & Novotny, 1986). Specific components have been identified that are associated with puberty acceleration (Vandenbergh, Finlayson, Dobrogosz, Dills, & Kost, 1976), the presence of estrogen (Schwenede, Wiesler, & Novotney, 1984), testosterone (Novotny, Schwende, Wiesler, Jorgenson, & Carmack, 1984), the promotion of inter-male aggression (Novotny, Harvey, Jemiolo, & Alberts, 1985) and male dominance (Novotny, Harvey, & Jemiolo, 1990). Additional components may be associated with an individual's diet and state of health (Brown & Schellinck, 1992). Consequently, it seems likely that the volatiles which signal the MHC genotype, along with other available cues are incorporated into an odour profile which is unique to each individual.

4.8 Behavioural evidence for MHC-generated cues in rats

The evidence for the presence of MHC-generated urine odours in rats had been provided by Brown and his colleagues (e.g. Singh, Brown, & Roser, 1987; Brown, Singh, & Roser, 1987). The majority of these findings have been in agreement with those on mice reported by Yamazaki and his colleagues although the work on rats concentrated more upon determing the mechanisms by which the MHC differences were expressed. The discovery by Singh et al., (1990) that bacteria are involved in the expression of odours of individuality may represent a major breakthrough in this area; nonetheless, it also creates an incongruity between the experiments with rats and mice that remains to be resolved. The investigations into the discriminability of urine odours in MHC congenic rats are summarized below.

4.8.1. MHC-based discrimination in a habituation-dishabituation task

The role of the RT1 complex in creating a unique odour profile was examined by Singh, Brown and Roser (1987). In an immunochemical analysis of class I MHC antigens (RT1.A), they determined that the RT1 molecules were present in soluble form in the lymph, serum and urine of rats. To determine whether urine containing these molecules could produce discriminable odours, they tested rats in a habituation-dishabituation task. Male rats of the PVG.RT1^{u} strain were able to discriminate between urine odours from PVG and PVG.R1 males which differed only at the classical class I MHC locus.

It was also determined that the urine odours of individual PVG and PVG.R1 males could be discriminated by PVG.RT1^{u} males as well as by Wistar albino and Lister hooded rats. Unlike the PVG.RT1^{u} rats, the Wistar and Lister strains did not share common background genes with the odour donors. Their ability to discriminate between these

odours eliminated any suggestion that the genetic similarity between the PVG.RT1^u, PVG and PVG.R1 rats could account for the test subjects' ability to detect differences between the urine odours.

Control tests were run in which PVG.RT1^u subjects were required to discriminate between odours from individual donors of the same strain as well as between odours from the same individual. Half the subjects were tested on the PVG strain and half on the PVG.R1 strain. Urines from the same individual of either strain could not be discriminated nor could different individuals of the PVG strain; however, individual males of the PVG.R1 strain were discriminated. In a retest, the urines from individuals from the PVG.R1 strain were not discriminated. It was suggested that the previous result may have been caused by hormonal differences or contaminants in the urines.

Having determined that individuals which differed only in the class Ia gene produced a discriminable odour which was volatile, Brown, Roser, and Singh (1989) carried out a detailed analysis of the discriminability of odours from rats congenic for different areas of the MHC (Figure 3.1). They found that male PVG.RT1^u rats, in a habituation-dishabituation task, could discriminate odours from pooled samples of urine from rats which differed in the following ways: 1) only in the class Ia region (confirmation of Singh et al, 1987); 2) in the two class 1 regions and the class II region of the MHC; 3) only in the class II B/D region; 4) and in all regions except class Ia. In all instances, control trials revealed that pooled samples made up from two groups of four different individuals of the same strain could not be discriminated.

4.8.2. The expression of MHC-based odours in rats

Singh, Brown, and Roser (1989) demonstrated that the discrimination of urine odours from PVG and PVG.R1 rats is dependent upon a volatile component in the urine. When

volatiles were removed from the urine by nitrogen purging, test subjects could no longer discriminate between the urines of male PVG and PVG.R1 rats in a habituation-dishabituation task.

In a further investigation, Brown, Singh and Roser (1987) examined the ability of adult males of three different strains of rats (PVG.RT1^{U}, Wistar and Lister) to discriminate a variety of odours associated with MHC molecules. They determined that neither serum odours of individual MHC congenic PVG and PVG.R1 rats nor saline-containing intact class I molecules purified from the urine could be discriminated by the test subjects. However, the fraction of the urine remaining after the intact class I molecules had been removed was discriminable. The authors suggested that these results could be interpreted in one of two ways: 1) either a fragment of the class I molecule or a small molecule associated with the class I molecule creates a discriminable odour, i.e., the carrier hypothesis, or 2) in accordance with a hypothesis by Schwende et al. (1984), the MHC may produce "variations in the pattern of general secondary metabolites rather than differences in the production of specific compounds". Klein (1986) also speculated that MHC-related odour discriminations were a result of odour differences produced by quantitative metabolic variations. In order to substantiate Brown et al.'s (1987) first hypothesis, all of the class I fragments should be removed from the urine and the remainder fraction tested for its discriminability. If the urine still produces odours which can be discriminated, it is unlikely that the MHC molecule, itself, plays a direct role in odour discrimination. This experiment has not yet been done.

Evidence from chemical analyses which have found mainly quantitaive variations between the urines of MHC congenic mice would appear to support the second hypothesis (Schewende et al., 1984; Tsuchiya et al., 1992). Because of their influence upon various physiological processes, different regions of the MHC as well as MHC-linked genes have

the potential to influence the volatiles in the urine (see Chapter 3). Behavioural tests which investigate the discriminablity of urine from MHC deficient class 1 and class II mice should provide definitive information regarding the role of the MHC in producing odours of individuality.

Singh et al. (1990) investigated Howard's (1977) hypothesis that the odour differences in MHC congenic strains of mice may not be related to the MHC molecules themselves but rather to differences in types of bacterial flora regulated by the MHC genes. They used a habituation-dishabituation task to examine the ability of rats to discriminate the urine odours of male rats reared in germfree and conventionally housed conditions. Test subjects (PVG.RT1^{u} males) were able to discriminate between the odours from pooled urine samples of conventionally housed male PVG and PVG.R1 individuals, but they could not discriminate between the urine odours of individuals of the same two strains which had been born and raised in germfree conditions.

To confirm these results, the experiment was repeated with a different strain of test subjects. These Lister hooded rats could also discriminate between the conventionally housed donors but not between the germfree donors. The possibility that the discriminability was a result of differences in the amount of MHC class I molecules produced by germ-free and conventional animals was considered. However, an enzyme immunoassay revealed that the titre of MHC molecules was identical in samples of urine from conventionally housed and germfree rats of the same strain.

Further verification of the importance of bacteria for producing individually unique urine odours in donors with different class I MHC genes was provided by gradually recolonizing the germfree rats with bacteria and moving them to conventional housing. Urine collected from these individuals was discriminable in a habituation-dishabituation

task, thus, confirming that bacteria are necessary for the production of individual odour differences.

In the process of introducing bacteria to the germfree animals, they were first innoculated with a cocktail of specific pathogens. To determine if these specific pathogens were sufficent to create a discriminable odour, urine was collected from PVG and PVG.R1 animals housed in a specific-pathogen free unit before they were fully conventionalized. In the habituation-dishabituation task, neither male Lister rats nor PVG.RT1^{u} could discriminate between urine samples from pooled individuals of these SPF PVG and PVG.R1 rats. When these animals were moved to conventional housing, pooled samples from the two strains could be discriminated. These findings would indicate that a variety of bacteria may be necessary for the odours of individuality to be expressed.

Singh et al. (1990) suggested that the results from this series of experiments could be interpreted in one of two ways: Either bacteria alone determine the uniqueness of odours and the presence of MHC antigens in the urine is irrelevant to the discrimination of individual odours or, alternatively, the MHC molecule associates with the volatile metabolites of bacteria and delivers these to the urine where they present a specific odour profile. If bacteria alone are responsible for individual odours, this would require every individual to have a unique population of bacteria. Singh et al. (1990) considered this to be unlikely and suggested that specific bacterial metabolites are carried to the urine in the peptide-binding groove of the MHC glycoprotein and released producing an odour unique to each individual. While the peptide-binding groove of each MHC molecule is somewhat specific, it is still capable of binding an enormous diversity of peptides (Englehard, 1994). Consequently, the hypothesis that it selectively binds bacterially-produced volatiles is quite speculative.

4.9. Behavioural evidence for MHC-generated cues in humans

There has been one investigation regarding the discriminability of the urine odours of humans who differed in their HLA type (Ferstl et al., 1989). Rats trained in a an olfactometer were able to discriminate between the odours of two tissue typed individuals which differed in a and b alleles of their class Ia genes. After learning the discrimination, the rats were tested for their ability to generalize the discrimination to the odours of other individuals with similar HLA-types. Initially, the rats did not discriminate between the odours from other individuals. With additional training on more HLA pairs, the rats did generalize their responses to other novel pairs of odours, thus, demonstrating that humans who have similar HLA types have a similar odour. The genetic background of the individuals with similar MHC types varied. Such a factor may have contributed to the initial difficulty of the rats in the transfer of training trials.

Humans may also be able to discriminate between the odours of individuals by using MHC-associated cues. Approximately 40 percent of individuals with a similar HLA type were identifed by a group of female volunteers as having intense body odours (Ferstl et al., 1989). Serum analysis has revealed that individuals with these specific HLAs have high concentrations of serum soluble class I molecules in comparison with other HLA types. The authors suggest these concentrations may result in a more noticeable body odour.

4.10. Summary

The main focus of this chapter has been a review of the behavioural studies which have examined the role of the MHC in the production of odours of individuality in mice and rats. Despite a number of procedural and statistical shortcomings, the mate choice studies

conducted by Yamazaki and his colleagues have provided evidence that male mice do discriminate between females on the basis of an MHC strain difference. Studies by other investigators have shown that females may also use MHC-based genetic cues to choose a mate. The odours of many variants of MHC congenic mice have also been discriminated by mice in a Y-maze and rats in a habituation-dishabituation task and in an operant-olfactometer. The results of these studies were generally in agreement although the finding that germfree rearing in rats removes the odours of individuality has not been replicated in mice. Apart from the hypothesis that bacteria are involved in the production of odours of individuality, no particular mechanism has been put forth to explain the way in which the MHC-based odours are expressed.

CHAPTER 5

THE ROLE OF BACTERIA IN THE PRODUCTION OF ODOURS OF INDIVIDUALITY

A vast population of microorganisms inhabits the skin surface and body cavities of mammals (Bibel & Lovel, 1976; Savage, 1977). As these microorganisms do not generally cause disease, they have been termed normal or indigenous flora. The presence of normal flora has been implicated in the production of chemical signals (Albone, 1984) and most recently, gut bacteria have been demonstrated to be necessary for the production of urine odours of individuality in rats (Singh et al., 1990). Such chemical signals are believed to originate from volatile compounds produced by the biochemical action of bacteria upon dietary and host substrate. This chapter provides an overview of bacterial metabolism of carbohydrates, protein and fats and discusses the role of dietary and genetic factors in influencing this process. It concludes with a review of the behavioural studies which have examined the effect of bacteria and diet in producing discriminable odours.

5.1. Characteristics of gut bacteria

The observation that the gastrointestinal (GI) tract was the natural habitat of bacteria was made in 1905 (Dubos, Schaedler, Costello,& Hoet, 1965). Nonetheless, it was not until the development of *in vitro* techniques for the culture of anaerobic bacteria, that the specific nature of these bacteria was determined. Prior to the use of such procedures, it was thought that bacteria were mainly found in the large intestine and consisted only of the facultative aerobe, *Escherichia coli.* (*E. coli*) (Savage, 1977). Subsequently, it was found

that anaerobic bacteria greatly outnumbered *E. coli* and were present throughout the gastrointestinal tract of many mammals (Savage, 1977). Estimates of the counts of total bacteria per gram of tissue have numbered in the millions (Dubos, Schaedler, Costello & Hoet, 1965; Smith, 1965; Raibaud, Dickinson, Sacquet, Charlier & Mocquot, 1966). The large intestine of both humans and laboratory rodents contains over 10^{11} micro-organisms per gram of intestinal contents (Finegold, Sutter, & Mathisen, 1983). Although up to 400 different species of bacteria have been isolated in the fecal matter of humans (Finegold et al., 1983), bacteria within the gut are frequently only identified at the genus level.

At this level of analysis, the bacterial population of many different species of animals appears to be quite similar. Smith (1965a) examined the stomach, small intestine, and caecum of 20 different species of animals including monkeys, dogs, cats, sheep, horses, rats and mice. Overall, the most common genera found were *Lactobacilli*, *Streptococci*, *E. coli*, *Clostridium welchii*, and *Bacteriodes*. The strains and numbers of bacteria found in individuals of the same species were similar to each other but different from those in other species. As each species was maintained on a different diet, it is possible that this difference accounted for dissimilarities in bacteria. Nonetheless, even within a species, different genera of bacteria predominated in different areas, for example, in rats and mice, *Lactobacilli* were found in different numbers throughout the GI tract but *Bacteriodes* were only present in the large intestine (See also, Dubos et al., 1965; Raibaud et al., 1966).

The development of bacterial populations in young animals has also been studied (Smith & Crabbe, 1961; Smith, 1965b, Savage, 1977). While the gut of the normal fetus is sterile (Gordon & Pesti, 1971), the fetus immediately acquires bacteria from the vagina and external genitalia of the mother as well as the external environment as soon as it is born. Van der Waaij (1986) has suggested that in humans the colonization may be divided into four phases depending upon the nature of the bacteria present: (1) from birth to two

weeks of age, the type of bacteria present are unstable (2) from two weeks of age to weaning, stabilizing bacteria are present and their nature depends upon whether the infant is breast fed or given formula; (3) after weaning, the bacterial population again becomes unstable because of changes in diet; (4) finally, establishment of the adult bacteria complete with colonization of the gut mucosa and degradation of intestinal mucous. The development of bacteria in rodents appears to follow a similar course (Schaedler, Dubos & Costello, 1965; Savage, Dubos, & Schaedler, 1968; Morishita & Miyaki, 1979).

Once established, bacteria occupy not only the lumina of the GI tract but also colonize the mucosal epithelium and the crypts of Lieberkuhn in the mucosa. The nutritional and environmental conditions vary in each of these areas; consequently, different types of bacteria have adapted to each habitat in the GI tract (Savage, 1977). The relationship between gut bacteria and their host organism is complex. In some instances, the relationship is beneficial to both the body and the microflora; in many cases, however, the bacteria compete with the host for nutrients to fuel their metabolic activities (Alcamo, 1994). It is the end products of these biochemical processes which enter the circulation and are excreted by the kidney as urinary volatiles. Which volatiles are produced depends upon both the nature of the substrate, i.e., carbohydrates, fats, or proteins, and which metabolic pathways are utilized. The following summary of bacterial metabolism illustrates the diversity of compounds which are found in the urine.

5.2. Bacterial metabolism

Metabolic processes may be divided into two categories: anabolism, the synthesis of compounds and catabolism, the digestion of chemical compounds. Catabolism provides bacteria with energy for growth and maintenance of cellular functions and leads to the production of volatile end products.

The metabolic significance of gut bacteria was established in the 1960's and 1970's by a number of investigators who compared the effect of germfree and conventional rearing on the constituents and end products of the GI tract of experimental animals (reviewed by Salter, 1984, Coates, 1984b, Eyssen & van Eldere, 1984). In general, the outcome of these studies established that bacteria catabolize carbohydrates, proteins and fats (Macfarlane & Cummings, 1991; Alcamo, 1994). While metabolism was found to occur in each section of the GI tract, because of the richness of the substrate and the relative stasis of the large intestine, the majority of reactions take place in the right colon . The following account of bacterial metabolism is summarized from Hentges (1983) and Macfarlane and Cummings (1991) unless otherwise specified.

5.2.1. Catabolism of carbohydrates

Both dietary and host substrate provide carbohydrates for bacterial metabolism. Dietary carbohydrates include starch, pectin, cellulose and sugars. The host organism contributes sloughed mucosa cells as well as glycoproteins produced by saliva, gastric juice and the mucinous secretions of the intestinal mucosa (Salyers & Leedle, 1983). Upon encountering bacterial enzymes from polysaccharide hydrolyzing bacteria, both external and host substrates are chemically broken down into less complex sugar molecules. At this point in the process, bacteria which had been unable to hydrolyze polysaccarides, also participate in metabolism of the carbohydrate fragments. This cross-feeding process maintains the diversity of bacteria and contributes to the variety of end products formed. The end products will also vary depending upon whether the bacteria which are involved in the process are aerobic or anaerobic.

The predominantly anaerobic bacteria of the large intestine produce ethanol, short chain fatty acids (SCFA), mainly acetic, propionic and butyric acids as well as the gases,

hydrogen, carbon dioxide and in some instances, methane. Other organic acids such as lactic and succinic acid may be final products of metabolism or further fermented to SCFAs depending upon the species of bacteria which are present to metabolize them. The volatile SCFAs are found in the urine as ketone body metabolites such as β-hydroxy-butyrate and acetoacetate. Many of these compounds are also produced by the action of bacteria on the secretions from the anal glands of small mammals (Albone, 1984).

5.2.2. Catabolism of proteins

The protein substrate which forms the basis of bacterial nitrogen metabolism is supplied by dietary components as well as by pancreatic and intestinal secretions and sloughed epithelial cells. The bacteria which utilize peptides and amino acids for energy are generally distinct from the carbohydrate fermenting bacteria. Nonetheless, in many instances, these bacteria also produce hydrogen, carbon dioxide, methane and similar SCFA end products such as propionic, butyric and acetic.acids. Additional SCFAs including isovaleric, isobutyric, 2-methylbutyric and oxaloacetic acids are also produced.

A variety of phenols and indoles are created by the degradation of the aromatic amino acids, phenylalanine, tyrosine and tryptophan. Amines such as dimethylamine, histamine, cadaverine and putrescine are also among the products formed. In addition, protein catabolizing bacteria are the major source of ammonia in the body. All of these products are excreted in the urine and the presence of urinary phenols and indoles has been used as a marker of gut bacterial activity (Greenberger, Saegh, & Ruppert, 1968; Bakke & Midvedt, 1970). It is interesting to note that phenol which has been produced by gut bacteria has been identified as the source of a sex attractant in the insect, *Costelytra zealandica* (Hoyt & Osborne, 1970).

5.2.3. Catabolism of fats and steroids

The digestion of fats leads to the production of long chain fatty acids and the subsequent formation of the steroid, cholesterol in the liver. Cholesterol and its primary bile acid derivatives, cholic acid and chenodeoxycholic acid provide metabolic substrate for the intestinal microflora. As many as 20 secondary bile acids including deoxycholic acid and lithocholic acid are generated by bacterial action upon these compounds and it has been suggested that the catabolism of bile acids is a major activity of gut bacteria (Hylemon & Glass, 1983). These compounds are either excreted directly in the feces or absorbed from the intestine, returned to the liver and eventually excreted in the urine.

Bacteria also participate in the metabolism of steroid hormones such as progesterone, cortisol, aldosterone, estradiol and testosterone. Like cholesterol, these hormones have been released into the gut from the liver. The majority of steroid hormone metabolites transformed by bacteria are returned to the liver via the enterohepatic circulation and excreted in the urine. A number of studies have indicated that the urine odours of individuals with different levels of hormones are discriminable (e.g. Landauer et al., 1977; Taylor et al., 1982; Johnston, 1983). It is possible that quantitative differences in the end products of bacterial metabolism provide the basis for this discrimination.

5.3. Influence of diet on the composition of gut microflora

The effect of changes in diet upon the composition of the gut microflora has most frequently been studied in rodents and humans (Smith, 1965; Drasar et al., 1973). In general, the increase in the detailed knowledge of dietary influences on gut microflora has paralleled the increased sophistication of microbiological techniques. Porter and Rettger (1940) reported that quantitative changes in *Lactobacilli*, yeast-like fungi and "other forms,

mostly coliforms" were produced in the stomach, small intestine and cecum of white rats by each of 12 different diets. Similarly, Dubos, Schaedler and their colleagues found that dietary differences resulted in quantitative and qualitative changes in the gastrointestinal *Lactobacilli* flora of white mice (Dubos & Schaedler, 1962; Dubos, Schaedler, Costello, & Hoet, 1965). Smith (1965) identified diet-dependent qualitative and quantitative changes in colonies of *E. coli, Cl. welchii, Streptococci, Lactobacilli,* yeasts and *Bacteriodes* throughout the gut of white rats.

The identification of changes in the number and specific type of bacteria resulting from a change in diet has been most in extensive humans. Holdeman et al. (1976) found significant quantitative changes in 7 of 15 species of bacteria tested in individuals before and after a change in their diet. Mevissen-Verhage et al. (1987) compared the flora of breast-fed and formula-fed infants. While *Bifidobacterium* and *Bacteriodes* were found in both instances, the species present within each genus proved to be quite different.

This latter finding illustrates the need to assess microflora very carefully in such comparative studies and appears to explain the inability of some early investigators to find a relationship between diet and the type of bacteria in the gut (e.g. Attebery, Sutter, & Finegold, 1972 cited in Finegold et al., 1983); Bounous & Devroede, 1974). They may have failed to find or were unable to test for subtle differences in the composition of the bacteria. Current studies utilize multiple stage continuous culture systems in an attempt to reproduce the physical and nutritional characteristics of the gut (Macfarlane, Hay, & Gibson, 1989). Using such a system, Gibson and Wang (1994) found that changing the carbohydrate growth substrate produced quantitative changes in a number of genera of fecal bacterial including *Bifidobacteria, Lactobacilli, Bacteriodes, Clostrida,* coliforms and gram positive cocci.

It is important to note that regardless of whether the diet specifically changes the qualitative nature of the microflora, its constituents will influence which metabolic pathways of the bacteria predominate and consequently affect the type of volatile end products released in the urine and feces. Such a result was described by Gibson and Wang (1994) who found that the addition of oligofructose to the dietary substrate in an *in vitro* simulation of gut action not only increased the numbers of *Bifidobacteria* but also caused quantitative and qualitative changes in the production of volatile short chain fatty acids.

5.4. Influence of genetic differences on the composition of gut bacteria

It has been suggested that differences in the composition of gut bacteria among individuals may have a genetic basis. This hypothesis was directly tested in a controlled environment by analyzing the fecal bacteria of three astronauts (Holdeman, Good & Moore, 1976). These individuals were isolated in the Skylab chamber and maintained on the same diet. A cultural count of several samples from each individual revealed qualitative differences in fecal bacteria among the three individuals. In addition, an analysis of the 15 most frequent species revealed significant individual differences in the frequency of 7 species. Still further proof that the bacterial flora are influenced by genetic differences comes from a study which determined that the anaerobic fecal flora of monozygotic twins are not significantly different whereas those of dizygotic twins differs (Van de Merwe, Stegeman, & Hazenberg, 1983)

There does not appear to have been any assessment of species specific differences in bacteria among individuals; however, the presence of methane producing bacteria may have a genetic basis. Bond, Engel and Levitt (1971) found 84 % of siblings of methane

producers also had methane on their breath compared with 18 % of siblings of nonproducers. Although these individuals have large numbers of methanogenic bacteria, they appear to have fewer sulfate reducing bacteria (Gibson, Macfarlane, & Cummings, 1988).

The relationship between genetics and type of gut bacteria of rodents does not appear to have been studied in any systematic manner although it has been reported that the biochemical activity of urea producing bacteria is influenced by the strain of mouse examined (Brockett & Tannock, 1982). Nonetheless, if Howard's hypothesis (1977) that the acquisition and stability of normal gut bacteria is controlled by the MHC is accurate, it would follow that all of the investigations into the role of the MHC in producing odours of individuality have been demonstrating the relationship between a particular gene complex and bacteria.

There is no doubt that the normal flora of the gut could not survive if they were presented by the MHC molecule as foreign antigen for processing by T cells. Consequently, some connection might exist between the tolerance of the immune system for specific gut bacteria and an individual's MHC type. Foo and Lee (1974) hypothesized that normal microflora must be related to the host's antigens in such a way that they are recognized as self. A review of the literature, however, does not reveal any specific research which has investigated the link between an individual's MHC type and the presence of a specific bacterial profile.

Several experiments have indicated that the intestinal flora which colonize the gut are specific to each individual and do not elicit an immune response in that individual. For example, inoculation of mice with their own bacterial flora compared with non-indigenous bacteria provokes a poor immune response to some bacteria and no response to others (Foo & Lee, 1972; Berg & Savage, 1975; Berg, 1983). Also, examination of fecal

bacteria of mice has revealed that normal flora are not covered with IgA antibodies whereas non-resident gut bacteria are (Van der Waaij, 1986). Moreover, it is not possible to maintain mixtures of intestinal bacteria of one individual in the gut of another without continuous inoculation (Van der waiij, Vossen, Altes, & Hartgrink, 1977; Lidbeck, Gustafsson, & Nord, 1987).

It has been suggested that tolerance for the normal microflora of the intestine may be a result of cross-reactivity between histocompatibility antigens and antigens on normal flora (Van der Waaij, 1986). This hypothesis is based upon the findings that cross-reactions have been detected between human HLA and streptococcal bacteria (Rapaport, Markowitz, Ralsbeck, Ayvazlan, & Balner,1972) and that HLA antigens are found on various types of bacteria (Prendergast, McGuignan, Gesczy, Kwong, & Edmonds; van den Broek, van dePutte, & van den Berg, 1988)

Currently, the association between the gut-associated lymphoid tissue (GALT) and the presence of intestinal microflora is being investigated (van der Waaij, 1986; 1991). Most recently, Woolverton, Holt, Mitchell and Sartor (1992) compared the development of T cells in the GALT of germfree and conventional mice. They concluded that the T cells of the GALT proliferate in response to intestinal bacteria and their products and that intestinal bacteria are responsible for the induction of an individual's immune competency. A similar experiment indicated that lymphoid cells in Peyer's patches are stimulated by specific apathogenic segmented filamentous bacteria (Klaasen et al., 1993). It would be speculative to suggest that the MHC glycoproteins may induce tolerance for specific gut bacteria by presentation of bacterial antigen to T cells in the developing GALT in much the same way as self-peptides are presented to T-cells in the developing thymus. If such could be shown to be the case, a strong argument could be made for a relationship between the MHC, gut bacteria and odours of individuality.

Recently, the development of high resolution nuclear magnetic resonance spectroscopy has provided the methodology to systematically identify urinary metabolites (Bell, Sadler, Morris, & Levander, 1990). It should be possible to use this technique to determine how both dietary and genetic factors alter the metabolic profile of urine. In so doing, one should be able to assess how rats and mice are discriminating between different urine samples. It would be particularly useful to analyze the urine collected from germfree and conventionally housed MHC congenic rats on the same and different diets to determine how genetics, diet and bacteria might interact to contribute to the synthesis of discriminable end products.

5.5. Behavioural evidence that bacteria are important in body odour production

Albone (1984) has summarized the role of microorganisms in producing odours in the skin, axillary glands, anal glands, vagina and oral cavity of mammals but the behavioural relevance of these odours is largely unknown. Moreover, while odours from urine and scent glands of rabbits, ungulates, carnivores and primates may be of bacterial origin (See Chapter 1.3.3), direct evidence of the role of bacteria in their production has rarely been demonstrated. Rather than investigating this hypothesis, behavioural scientists seem to take this knowledge for granted (e.g. Clark, 1978; Gubernick, 1980). The results of several studies which have provided either indirect or direct evidence for the relevance of bacteria in the production of individual odours are described below.

Studies which have provided the most substantive evidence for a bacterial basis of individual odour are those which have conducted chemical, bacteriological and behavioural analyses of the anal sac secretions of the Indian mongoose (Gorman et al., 1974). These analyses determined that there were quantitative individual differences in the volatile

components of the anal sac and that bacteria were necessary for the production of discriminable differences in the secretions of the anal gland.

Based upon these results, Gorman (1976) formed a fermentation hypothesis which postulated that a consistent individual odour profile was created by the action of different bacterial populations on a chemically stable glandular secretion. He suggested that the presence of this unique odour was the basis of individual recognition. A long term analysis of the secretions of an individual as well as behavioural evidence of an inability to discriminate between bacteria-free secretions would be required to substantiate this hypothesis. Moreover, if the fermentation hypothesis were of general significance in individual recognition, one would expect to find chemical and behavioural evidence regarding the relevance of these anal gland secretions in other species of mammals. However, an extensive analysis of the anal gland secretions of the red fox by Albone (1984) indicated that the differences within individuals over time were as great as differences between individuals.

In humans, identification and categorization of the bacteria present in the skin has shown that there are both qualitative and quantitative differences in bacterial populations between individuals (Bibel & Lovel, 1976). The distribution of bacteria over the skin surface of each individual is unique and creates what Bibel and Lovel call a skin microflora map. While it is not likely that these odour differences are used in individual recognition by humans, there is some evidence that they could serve such a function. Wallace (1977) found that women could discriminate between the perspiration-induced hand odours of individual unrelated females.

Porter and Moore (1981) determined that parents could discriminate between the body odours of two of their children using olfactory cues contained in the children's T-shirts. As the odour component of human axillary secretions is bacterially determined, the ability

to discriminate between the individual odours of humans on the basis of their axillary odours may be due to bacterial action. Since the ability to discriminate between bacteria-free axillary secretions has not been tested, however, one cannot make firm conclusions about the role of bacteria in producing these odours.

In other mammals, bacterial odours produced in the anal or urogenital region can serve as recognizable chemical signals. In rabbits, volatile fatty acids found in inguinal glands provide information about individual identity (Hesterman & Mykytowycz, 1982a; 1982b). Microbial analysis has revealed that a number of bacteria are present which are responsible for the production of these volatiles (Merritt, Goodrich, Hesterman, & Mykytowycz, 1982). In mice, volatile compounds originating from the feces of a male increase the heart rate of another male and also alter the marking pattern of the mouse within its home territory (Goodrich, Gambale, Pennycuik, & Redhead, 1990).

Vaginal bacterial flora may also produce olfactory cues which could be used to discriminate between females. In an investigation of such a phenomenon, Merkx, Slob and van der werff ten Bosch (1988) found that male rats showed a preference for an estrous female with an untreated vagina over an estrous female with an antibiotically sterilized vagina. It may be that these vaginal odour differences can only be used to discriminate between classes of females, such as estrous versus non-estrous, and not between individuals. Nonetheless, such odours would still contribute to an individual female's odour profile. Further behavioural assessment along with a comparative analysis of the bacterial content of the vaginal secretions of individual females is required to determine how relevant these bacteria are for individual discrimination in rats.

The action of bacteria has also been implicated in the production of a specific maternal pheromone in rats. Following the discovery that rat pups prefer the odour of a lactating female to that of a non-lactating female (Leon & Moltz, 1971), Leon (1974) investigated

the source of the odour. In a series of preference experiments, he determined that rat pups were attracted to the caecal contents or caecotrophe being synthesized and excreted in large quantities by lactating females. He hypothesized that caecal bacteria produce a maternal pheromone which is excreted in the caecotrophe.

In contrast to Leon's (1974) theory, Moltz and Lee (1980) suggested that the pheromone is not found exclusively in the caecotrophe and that its formation depends upon the ability of caecal bacteria to transform cholic acid into volatile metabolites. Regardless of the exact nature of the mechanism of synthesis, it seems clear that specific odours found in maternal feces are produced by the action of bacteria.

The presence of pathogens in the body also appears to influence the odours of individuals. Animals, including humans, that are sick produce odours which distinguish them from healthy individuals (Bedichek, 1960, Kavaliers & Colwell, 1993). Female mice infected with parasites are discriminated from healthy females by potential mates (Edwards & Barnard, 1987). Birds and fish which are infected with parasites are discriminated on the basis of their secondary sex characteristics (Zuk, Johnson, Thornhill, & Ligon, 1990; Milinski & Bakker, 1990). While the role of odours has not been directly examined in these studies, it is possible that the bacterially-produced volatiles found in urine and feces may also provide information regarding health status. Hamilton and Zuk (1982) suggest that the examination of urine during courtship rituals may be useful in helping animals choose mates that are resistant to disease. The odours of disease may also assist predators in locating their prey. Recently, dogs trained to locate birds by scent found significantly more parasite-infested red grouse than antihelmenthic-treated individuals (Hudson, Dobson & Newborn, 1992).

Definitive conclusions concerning the role of bacteria in the origin of individual odours requires a comparison of the discriminability of odours from germfree and

conventionally maintained individuals. As described in section 4.6.1, such an experiment was undertaken, using rats as subjects. Singh et al. (1990) found that rats tested in a habituation-dishabituation task did not discriminate between the urine odours of germfree MHC congenic rats. In addition, Schellinck et al. (1991) showed that although rats trained in an operant-olfactometer could discriminate between the urine odours of germfree MHC congenic rats within a single test session, they could not remember this discrimination from one daily test session to the next session. This pattern of learning was much the same as that of these same rats when given the odours of genetically identical individuals to discriminate. Thus, the authors concluded that the discrimination of the odours from the germfree individuals was based upon extraneous differences and that a constant odour of individuality was not present in such stimuli.

These latter results are contrary to those obtained by Yamazaki et al. (1990), in which mice were shown to be able to discriminate between the urine odours of germfree mice. Chapter 7 of this thesis is devoted to a further investigation of the discriminability of the urine odours of germfree mice. Chapters 8 and 9 assess the relative contribution of different types of bacteria to odours of individuality.

5.6. Behavioural evidence that dietary changes alter the nature of body odours

Neither the role of diet in altering the odours of an individual nor the mechanism by which it might act to do so has been widely examined. Galef and his colleagues (1989a; 1990) have consistently demonstrated that information about the familiarity of a diet is transmitted to rats through the body odours of their conspecifics; however, they have been interested in the social learning processes involved rather than how different diets changed the odours of the "demonstrator" rats. Several other investigators have changed the diet of an animal to specifically determine if this manipulation will result in discriminable

differences in the odours of individuals. A few of these have suggested that the differential action of bacteria upon the dietary substrate is likely responsible for the modification of an individual's odour (Leon, 1975, Skeen & Theissen, 1977).

Odours which originate from dietary factors may be transmitted in a variety of bodily secretions. Leon (1975) found that 16 day-old rat pups approached the odours from the anal excreta of unfamiliar lactating females on the same diet as their own mother significantly more often than the odours of unfamiliar mothers on a different diet. Beauchamp (1976) determined that the urine odours of adult guinea pigs on different diets could be discriminated by other guinea pigs. The ventral scent gland secretions as well as whole body odours and the bedding of gerbils maintained on either lab chow or dog food were discriminated by both preweanling and postweanling pups (Skeen & Thiessen, 1977).

Moreover, it appears that the ability to discriminate between the odours of individuals on different diets develops at a very early age. Cues contained in mother's milk influenced the diet choice of rat pups after weaning (Galef & Henderson, 1972; Galef & Sherry, 1973; Bronstein, Levine & Marcus, 1974). Most recently, Hepper (1988) has shown that the offspring of mothers fed garlic during pregnancy prefer the odours of garlic compared with the odours of onion. Such a finding would suggest that the ability to learn about dietary cues begins prior to birth. Whether the information is transmitted to the fetus though the blood or amniotic fluid has not been ascertained although the amniotic fluid is known to contain discriminable olfactory cues (Hepper, 1987).

The most extensive examination of the influence of changes in diet upon behaviour was undertaken by Porter and his colleagues. They initially observed that *Acomys cahirinus* mouse pups which had been fostered onto *Mus musculus* females associated more frequently with the body odours of lactating *Mus musculus* than with the odours of

lactating *Acomys* (Porter, Deni & Doane, 1977). The diets of the two species differed slightly in this study but as species-specific differences may also have existed, it was not possible to know why the pups responded as they did. Porter and Doane (1977) subsequently determined that spiny mouse pups preferred the odours of lactating females on the same diet as their own mother compared to the odours of conspecific mothers on a different diet. Moreover, the bedding odours from *Mus musculus* maintained on the maternal diet were preferred over the odours of unfamiliar diet conspecific. In this instance, dietary factors rather than species-specific cues appear to most significant in determining the response of pups to maternal cues.

It appears that the timing and length of exposure to different diet cues also influences the subsequent response to the odours. Spiny mouse pups which experienced the odours of their own mother for the first day of life preferred the odours of other lactating females on the same diets; however, when these pups were then fostered onto other diet females, they had no preference for odours from same or different diet mothers at four days of age (Doane & Porter, 1978). At six days of age, however, they preferred the odours of the different diet mothers.

Discriminable diet cues are also present in the odours of pups. Lactating females differentially retrieved the one-day-old pups of females maintained on the same diet as themselves (Doane & Porter, 1978). In addition, weanlings themselves prefer the odours of agemates on the same diet as themselves (Porter & Doane, 1979). Nonetheless, as other experiments have indicated that same sex littermates can also be discriminated (Porter, Wyrick & Pankey, 1978; Porter & Wyrick, 1979), dietary and genetic cues influence recognition cues in spiny mice.

To test this hypothesis, the huddling preferences of weanling spiny mice for kin and non-kin on same and different diets were examined (Porter, Mcfadyen-Ketchum, & King,

1989). Unfamiliar non-kin and unfamiliar kin on the same diets interacted more frequently than nonkin on different diets. Most interesting was the finding that familiar kin on the same diets huddled together more frequently than any of the latter groups. The authors concluded that diet and genotype contribute additively to recognition signatures in spiny mice. In contrast, to this finding, an examination of the preference of MHC congenic mice for the feces odours of mothers of the same or different strains on the same or different diets showed that diet but not genotype influenced the preference of pups (Brown & Wisker, 1991). Thus, it appears that the relative contribution of dietary and genetic factors in the production of odour cues is uncertain. Chapters 9-13 of this thesis further explore the role of a change in diet and genetic differences at the MHC in contributing to odours of individuality.

5.7. Summary

The presence of non-pathogenic bacteria is essential for the metabolism of carbohydrates, proteins, fats and steroids in the gut. Moreover, the end products of these catabolic processes provide the energy necessary for the survival of both bacteria and the host organism. The excretion of these end products results in the presence of discriminable volatiles in the urine and feces which may be responsible for the production of odours of individuality. Both genetic differences and changes in diet have been shown to produce quantitative and qualitative changes in bacteria which influence the subsequent discriminability of urine odours. Although a relationship between the MHC and gut bacteria and odours of individuality has been hypothesized, there is, as of yet, no empirical evidence to support this theory.

CHAPTER 6

METHODOLOGY AND HYPOTHESES TO BE TESTED

I used a habituation-dishabituation task and the operant-olfactometer to assess the discriminablity of urine samples from mice and rats. This chapter describes the origins of the methods as well as their methodological strengths and weaknesses. The chapter concludes with an outline of the hypotheses to be tested in this thesis.

6.1. The habituation-dishabituation paradigm

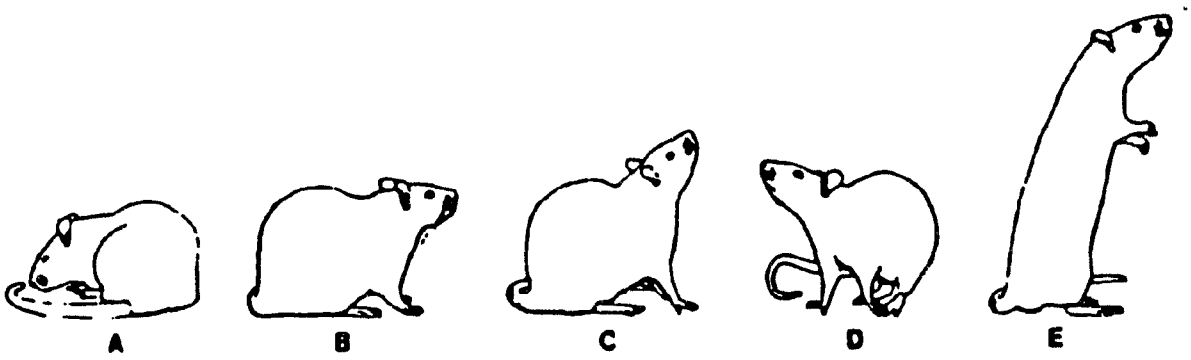
The habituation-dishabituation test is based upon the premise that successive presentations of the same stimulus odour to a subject will result in a decrease in behavioural responses to that stimulus, i.e., habituation. When a different odour stimulus is then presented to the subject, the habituated response is reinstated or "dishabituated". The change in time spent orienting toward and sniffing the odour is used as the criterion for determining olfactory discrimination.

Generally, each test begins with a no-odour adaptation period during which subjects are presented with the odour vehicle which has either been covered with water or left dry (e.g. Brown, Singh & Roser, 1987). The test session usually consists of brief consecutive trials in one daily session; however it may not be necessary to conduct the trials in this manner. Beauchamp and Wellington (1984) have shown that habituation occurs when individual urine odours are presented for only two minutes, seven days apart. Moreover, three days later when a novel odour was presented, dishabituation occurred.

The habituation-dishabituation test was first used to demonstrate the role of odours in individual recognition in flying phalangers (Schultze-Westrum, 1964) and lemurs (Harrington, 1976) and has subsequently been used in such diverse species as dogs and wolves (Brown & Johnston, 1981) and laboratory rats (Singh et al., 1987). Gregg and Theissen (1980) developed a simple method for testing olfactory discrimination in gerbils which could be adapted for use with many species of rodents. The test apparatus consisted of a rectangular Plexiglass box with a removable cover. Odours were presented on a cotton tipped applicator through a 1.25 cm hole in the centre of the cover. Each test session was divided into four, six minute periods. Responses to a dry applicator were recorded in the first period, followed by responses to different odours in each of the following six minute blocks. Within each block, the odour was renewed every minute. The authors found that both frequency and duration of rearing were accurate and reliable measures of discrimination but that overall activity level did not provide a reliable measure of discrimination.

Sundberg, Doving, Novikov and Ursin (1982) designed a test apparatus in which the odour was blown into the top of a circular chamber. Not only have they provided an excellent description of the orienting reaction of the rat to an odour (Figure 6.1) but they also categorized each stage of the response so that consistent measures of scoring could be applied across test sessions.

Brown and his colleagues (Brown, Singh, & Roser, 1987; Singh et al., 1990) adapted the method and apparatus of Gregg and Theissen (1980) for use with rats and measured the responses described by Sundberg et al. (1982). Their results as illustrated in Figure 6.2 demonstrate the typical pattern of rearing and sniffing at odours and the changes produced by habituation and dishabituation.



Fgure 6-1. The sequence of the orienting response of a rat to an odour presented from above. Before odour presentation the animal is at rest (A). When the odour is presented, the rat lifts his head and forepart of the body (B, C), moves about (D), and finally rears (E) (reprinted with permission from Sundberg *et al* ., 1982). Only the time spent rearing and sniffing at the odour stimulus was recorded in our studies.

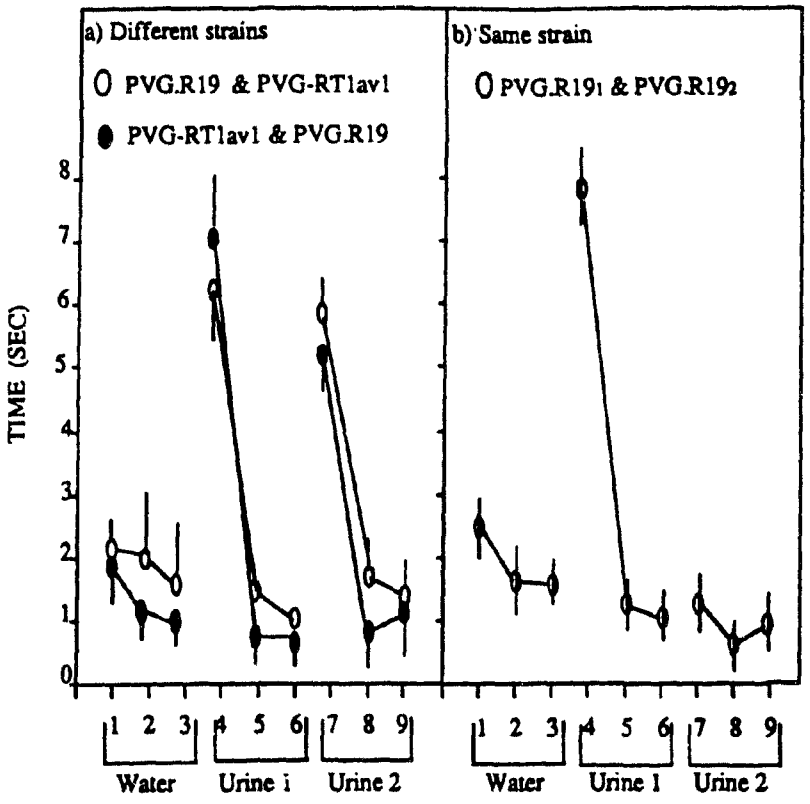


Figure 6-2 Mean (± sem) time (sec) spent rearing and sniffing at urine odours from (a) individuals from two MHC congenic strains (PVG-RT1^{av1} and PVG.R19) and (b) two individuals of the PVG.R19 strain. In (a) dishabituation occurred on Trial 7, indicating that the odours from two strains could be discriminated. In (b) the rats did not dishabituate when presented with a second urine sample from an individual of the same strain (redrawn with permission from Brown et al, 1989

6.1.1. Advantages and Disadvantages of the Habituation-Dishabituation Test

The principal benefits of the habituation-dishabituation tests arise from its simplicity of design and speed and ease of testing. This method requires no training of the subjects, measures a naturally occurring response and has been shown to be sensitive to subtle odour differences (Brown, Singh, & Roser, 1987). If dishabituation occurs, the results of the test are easy to interpret as this indicates a discrimination between the two odours.

The main drawback of the habituation-dishabituation test comes from the difficulty in interpreting a negative result. Does continued "habituation" really mean that the odours are indiscriminable or does the animal lack the motivation to continue the task or does he find the odours presented to him aversive? The answers to these questions are not readily apparent. In the determination of individual odour differences, this could limit the interpretation of the results unless the absence of discrimination is confirmed by another less ambiguous methodology.

6.1.2 Apparatus and Procedure for the Habituation-Dishabituation Test

The apparatus used for the habituation-dishabituation test consisted of a 38.5 X 32 X 17.5 cm opaque plastic box covered by a 38.5 X 32 X 9.5 cm wire mesh top, thus, making the cage 27 cm high. A 5.5 cm. circle of filter paper (Whatman #1) was taped to the outside of the centre of one side of the mesh top, so that the centre of the filter paper was about 22 cm from the floor of the box.

About 0.10 ml of liquid was drawn from a 1 ml syringe (Becton-Dickinson) and placed upon the the centre of the filter paper . Each odour presentation trial lasted two minutes, after which the top of the cage was removed and replaced with another top containing a new piece of filter paper. Trial length was timed using a digital count-down timer (Micronta). The used top was rinsed with water and allowed to dry prior to being

reused. A clean testing chamber was used for each subject. After use the boxes were rinsed with water, sprayed with 70 % ethyl alcohol and air-dried.

In the experiments presented in Chapters 8, 10 and 12, each subject received nine sequential two minute odour presentations. Water was presented on the first three trials to provide the subjects with a six minute habituation to the apparatus and the procedure. On the next three presentations, urine of one sample type was used and on the final three presentations, the type of urine presented varied. In an experimental trial, the urine samples would be of a different type than previously presented. In a control trial, the second sample would be the same type as the first.

Thus, while the top of the chamber was changed every two minutes, the type of odour stimulus changed only on presentations four and seven. To determine if the subjects could detect this change, an observer, blind to the conditions of the experiment, recorded the time spent by the rat rearing on its hind legs and sniffing or licking the odorized area of the filter paper. The observer used a stopwatch (Hurer) to record the cumulative time spent rearing and sniffing for each two-minute presentation. Tests were conducted under dim white light in the dark phase of the L:D cycle.

6.2. The operant-olfactometer paradigm

An operant-olfactometer consists of a test chamber and odour delivery system. The test chamber contains an odour port, a response key and a water delivery mechanism; the odour delivery system includes a clean air stream and a number of odour streams which are electronically controlled. The subject is required to make a successive discrimination between two or more stimuli by responding to the stimuli differentially. Reinforcement is available following a correct response. The design of such a system may vary depending

on how the odour stimuli are generated and delivered, how the test chamber is constructed and how the test animal is required to respond to receive a reward.

6.2.1. Development of olfactometry

The olfactometer designed by Pfaffman, Goff and Bare (1958) for the rat has provided the basis for many of the models currently in use. They constructed a manually controlled odour delivery system entirely from glass parts in which odorized air could be added to a clean air stream. The rat, situated in a "wind-tunnel", was trained to face into the air stream and to bar press for water reinforcement in the absence of the odorized air. This system was modified by a number of investigators (Goff, 1961; Braun, Wermuth, & Haberly, 1967) and culminated in the production of an automated olfactometer constructed of Teflon solenoid valves with individual flowmeters to monitor and control the stimulus airflow (Slotnick & Nigrosh, 1974).

In this apparatus, rats were first trained to obtain water reinforcement after they broke a photobeam in the front of the operant chamber; then, they were required to sample an odour which was delivered in the nose cone after the photobeam break prior to getting reinforcement. Finally, the rats learned to make a discrimination between several odours which were presented sequentially and to make an operant response only to the odour designated as the positive stimulus (S+).

Slotnick (1990) continued to modify his apparatus so that the delivery of odours is now controlled by a computer system. Also, after presentation of the stimulus, reinforcement is contingent only upon a licking response. Moreover, the odour stimulus no longer exhausts through the animal chamber; rather, the subject is trained to sample the stimulus airstream for a specified time prior to the odour being shunted to exhaust through a connection to the odour sampling tube. This further eliminates the possibility of any

residual odour remaining in the test chamber during the intertrial interval. Olfactometers based upon Slotnick's designs are currently in use in a number of behavioural labs (Doty & Ferguson-Segall, 1989; Beauchamp et al, 1985; Schellinck, Brown, & Slotnick, 1991) and have been used to assess the detection and discrimination capabilities of both normal rats and those with experimental brain lesions (Slotnick, 1990). This olfactometer has a multiple odour discrimination system. As well as providing the opportunity to investigate the response to as many as eight odours per session, this apparatus permits the presentation of mixtures of odours and the investigation of stimulus generalization. Lu, Slotnick and Silberberg (1993) have trained rats in a delayed matching to sample problem using this apparatus. A modification of this system will be used in the experiments described in Chapters 7, 9, 11 and 13.

6.2.2. Advantages and disadvantages of operant-olfactometers

The advantages of an operant olfactometer include the use of a contained system to prevent odour mixing and the accurate control over the stimulus concentration, duration and delivery. The use of flowmeters to monitor airflow also eliminates the possibility that discrimination is based on differential air flow or pressure cues. In terms of data collection, any problem of experimenter bias is eliminated as the subject controls the initiation of each trial and responses are usually automatically recorded.

The use of an operant response provides a quantitative dependent variable. Quantitative measures may be obtained not only of the animal's ability to discriminate between odours but also of the types of errors made while learning the discrimination and the time required to learn the discrimination. The high level of performance of the animals trained in this apparatus is also indicative of the suitability of the apparatus for studying olfactory learning (Slotnick, 1984) .

Moreover, by using an operant-olfactometer, the delivery of odours can be controlled. Thus, the most serious problem with using a Y-maze for discrimination studies, i.e., the potential inability to control the delivery of the odours, particularly if the stimuli are presented simultaneously, is overcome. Depending upon a number of factors, such as the effectiveness of the exhaust fan, the design of the maze, and the point at which the odours are sampled, the intensity and composition of the odour could change from trial-to-trial during maze testing (Slotnick, personal communication). Such methodological flaws may have contributed to the inability of Reid and Morris (1992) to replicate the learning set and reversal learning experiments carried out in an operant-olfactometer by Slotnick and his colleagues (e.g. Slotnick & Katz, 1974; Nigrosh, Slotnick, & Nevin, 1975; Slotnick, 1984).

Although a number of potential confounds have been removed by the use of automated equipment, it is still necessary to perform control trials to ensure that the subject is not basing his discrimination upon extraneous odour cues which have remained in the system. To test for contamination, a rat which has been previously well trained in the apparatus to discriminate between odours must be given test sessions in which odour stimuli have been replaced by clean, empty stimulus jars. If the rat can discriminate between the air samples from two "clean" channels, contaminating odours are present and the system must be flushed with 70 per cent ethanol and air dried until further testing with a rat reveals that no discriminable odour cues remain.

Acetone may be required to dissolve the residue left by biological fluids such as urine. While this is very effective, great care must be taken in using acetone, as its inhalation can cause respiratory and neurological damage in humans. On occasion, no amount of cleaning will remove residual odours and the apparatus must be methodically checked until the contaminated section of equipment is found and replaced.

One problem with investigating olfactory discrimination in this apparatus, as in any other method using operant conditioning, involves the possibility that the subject will be motivated to discriminate between odours that he would not discriminate in a more natural situation. If an experimenter is investigating an animal's ability to discriminate between biologically significant odours, the results obtained in an operant olfactometer must be evaluated with this fact in mind. Moreover, the decision to build an olfactometer will depend on a firm commitment to olfactory research of this type, as the initial cost of Teflon solenoid valves and glass flowmeters can be prohibitive. The construction of a properly functioning system is complicated and adherence to proper cleaning procedures requires an almost fanatical dedication.

6.2.3. The olfactometer

An olfactometer (Figure 6.3), based upon the original design of Slotnick and Nigrosh, (1974) was constructed for the controlled delivery of urine odours. Clean air was provided for this olfactometer by a continuous duty GAST compressor pump (Model MOA-P101-AA), [1] (Numbers in square brackets [ ] refer to components of Figure 6.3) and this air stream was filtered through activated charcoal contained in a 22 cm x 16 cm X 24 cm plastic battery box [2] Two fritted glass filters (custom made by Toms Scientific Glassblowing, Frederick, Maryland) [3] provided a final filtration of the air stream prior to its entry into the olfactometer.

The rate of air flow into the olfactometer was regulated by five Kontes # 2 needle valves [4] and was measured by Porter Instrument Company flowmeters. The main air flowmeter, model B-125-50 [5], monitored the main air flow and the other four flowmeters, model A-125-7 [6], measured the flow of air to each of four stimulus channels. The remainder of the olfactometer, as described below, consisted of a system of

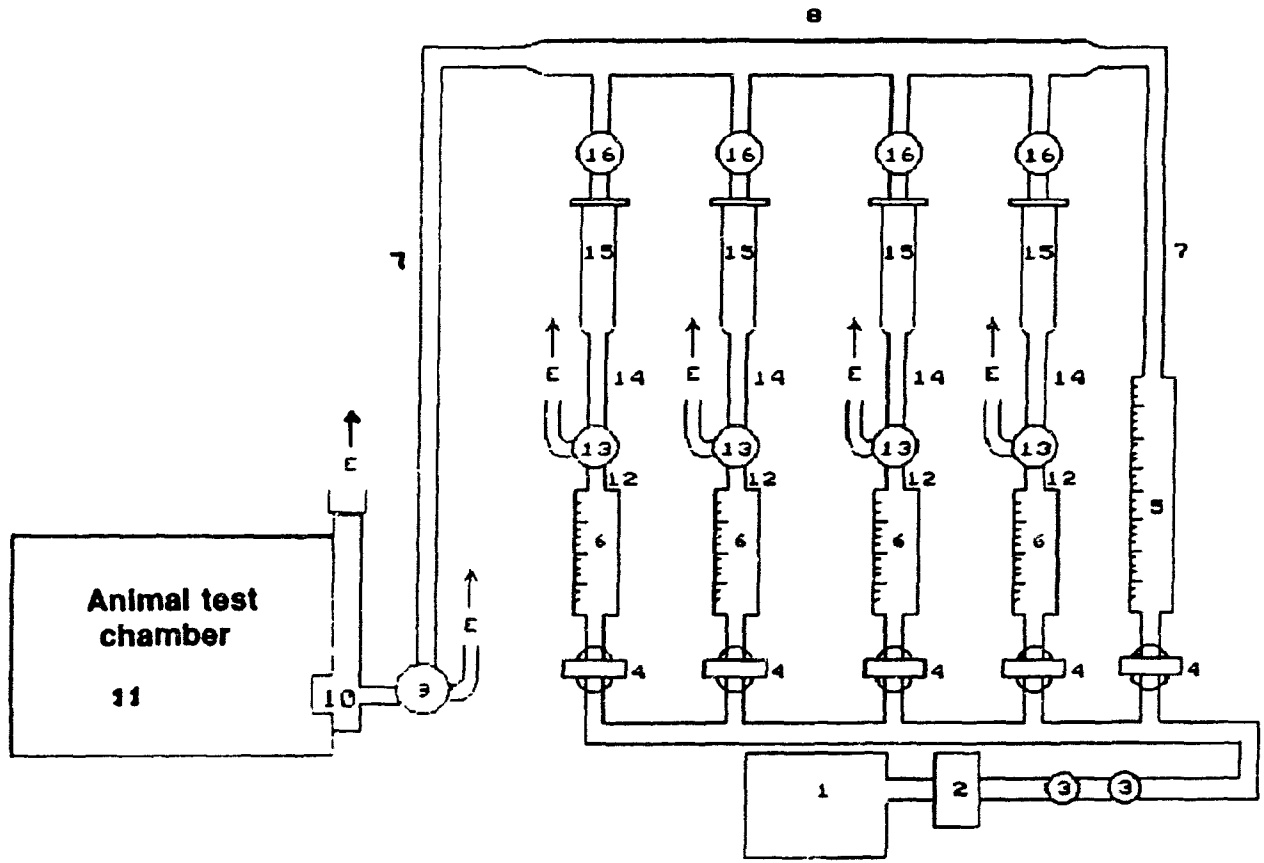


Figure 6.3. A schematic representation of the olfactometer. 1. Gast compressor pump; 2. activated charcoal filter; 3. fritted glass filters; 4. needle valves; 5. main air flowmeter; 6. stimulus channel flowmeters; 7. Eight mm. ductwork; 8. glass manifold; 9. final valve; 10. odour sampling tube; 11. animal test chamber; 12. Teflon connector; 13. 3-way solenoid valve; 14. Four mm. ductwork; 15. odour stimulus jar; 16. 2-way solenoid valve; E = to exhaust.

glass ductwork and valves which controlled the delivery of clean air and odorants to the test chamber.

The ductwork [7] which carried the main air stream through the system after it passed through the main air flowmeter [5] was constructed from 8 mm (id) glass tubing. The glass tubing was cut and handblown to the length and shape required to accomodate the other components of the system and joined with connectors cut from 8 mm (id) Teflon tubing (Cole Parmer Instrument Company, Chicago, Illinois). The glass tubing, with Teflon connectors, extended 130 cm from the main air flowmeter [5] to one of the inputs of the glass manifold [8]. The output of this manifold was connected with 20 cm of 4 mm (id) Teflon tubing to a glass adaptor leading to the common port of the final valve [9].

The final valve was a three-way Teflon solenoid valve, (Model 800, Mace Corp., El Monte, California), which controlled delivery of clean and odorized air to the test chamber. The normally open port of the final valve was connected to the bottom of a glass odour sampling tube [10] which opened directly into the test chamber [11]. The normally closed port of the final valve was coupled to a 2.5 m length of 8 mm rubber tubing which exhausted to an outside air vent [E]. Thus, when the final valve was open, clean air was delivered to the test chamber and when the final valve closed, the clean air was diverted from the test chamber to the exhaust line.

Each stimulus channel flowmeter [6] was joined by a Teflon connector [12] to the common port of a three-way solenoid valve (General Valve Corp. model 1-30-900) [13]. From the normally closed port of this 3-way valve, ductwork [14] constructed from 4 mm glass tubing connected by sections of 4 mm Teflon tubing delivered clean air to the stimulus channels. The glass tubing with its Teflon connectors extended approximately 80 cm, to the odour stimulus jar [15].

The stimulus jar used in the experiments described in Chapters 7 and 9 is shown in detail in Figure 6.4. The stimulus jars were made from 30 ml disposable plastic syringes (Becton Dickinson). The plunger was discarded and the rubber stopper [A] was connected to the outflow tube [B]. A cotton ball (Ingram & Bell 13-0050) [C] was placed in the syringe as a vehicle for the odorant. Clean air was blown into the syringe via 4 mm (od) glass attached to the tapered end of the syringe [D]. Odour saturated air exited through the glass ductwork which was pushed through a 4 mm opening in the rubber stopper and connected the stimulus jar to the input port of a 2-way solenoid valve (General Valve Corp. model 2-40-900) [16]; the output port of the solenoid valve was joined via a Teflon connector to the glass manifold [8] of the main air stream and thus to the final valve [9] and the odour sampling port [10]. The distance from the top of each odour stimulus jar [15] to the odour sampling port was approximately 45 cm. The odour stimulus jars could easily be removed from the ductwork to add the odorant or to replace the jars with those containing new odour stimuli.

The release of the odorant from each of the four stimulus jars was controlled by the action of the two solenoid valves [13 and 16] as follows: the common port of the three-way valve [13], upstream from the stimulus jar, received a continuous flow of clean air from the flowmeter [6] which was sent to exhaust through the normally open port. When this three-way valve closed, the clean air was diverted to the stimulus jar [15] where it became saturated with the odorant. Coincident with the operation of the three-way valve [13], the two-way valve [16], downstream from the stimulus jar, opened and the odour-saturated air was introduced into the glass manifold [8] to join the main air stream.

The action of the two stimulus valves was synchronous with the closing of the final valve [9]. When the final valve closed, the clean air stream to the test chamber was briefly (0.7 sec) sent to the exhaust line while the odour saturated air mixed with the clean air in

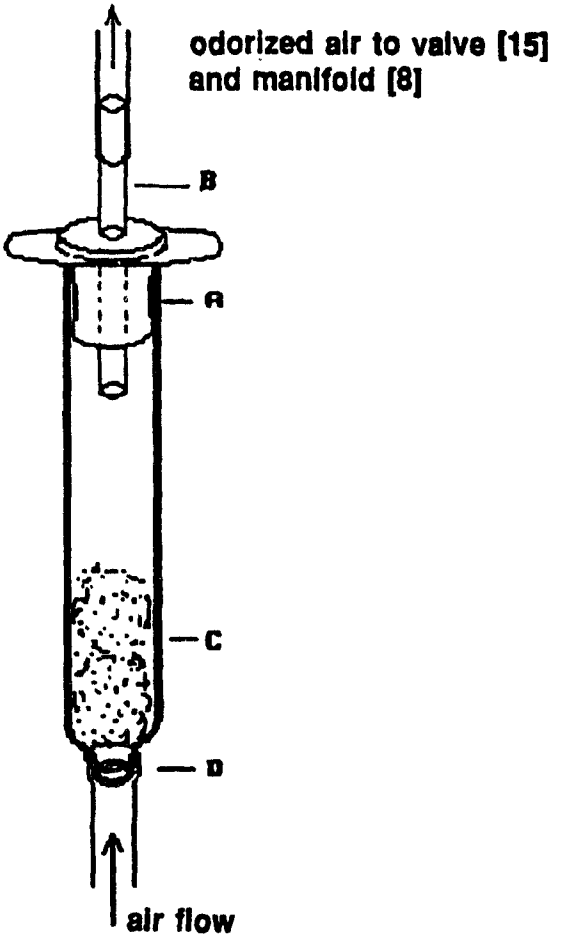


Figure 6.4. A schematic representation of the odour stimulus jar: A. rubber stopper; B. outflow tube; C. cotton ball; D. Teflon and glass ductwork

the manifold and was delivered to the final valve. When the final valve re-opened, this odourized stream was presented to the test animal through the odour sampling tube [10]. For the experiments described in Chapters 8, 11 and 13, a second olfactometer was constructed with the same specifications as described above. Several improvements were made to both systems, at this time. The plastic syringes used for stimulus jars were replaced with custom made glass tubes with an inport and an outport (Precision Glass Blowing, Dartmouth, N. S.). The odour delivery system was modified to increase the number of stimulus channels from four to eight. Also, a single stimulus channel flowmeter [6] joined by a Teflon connector [12] to the common port of a three-way solenoid valve (General Valve Corp. model 1-30-900) [13] was used to provide air to each stimulus channel. The two way valves (General Valve Corp. model 2-40-900) controlling the airflow into and out of the stimulus jars were replaced with newer models (General Valve Corp. model 3-507-900). The final valve (Model 800, Mace Corp., El Monte, California) was replaced by General Valve Corp. model 9-321-900. These valves were technically superior to those previously used but did not change the amount of airflow delivered throughout the system.

6.2.4. Animal Test Chamber

The animal test chamber (Figure 6.5) consisted of a 26.5 X 17 X 21.5 cm acrylic box [11] with a stainless steel grid floor (1.1 cm2) [17] and a removable acrylic latch top [18]. The animal's home cage, placed below the box, was used as a litter pan. One side of the test chamber contained the odour sampling port [10], a light source [19], a photocell [20] and a drinking tube [21].

The odour sampling tube [10], consisted of a vertical glass tube with a circular opening leading into the animal chamber. This tube was custom blown by Toms'

Scientific Glassblowing Co., Frederick, Maryland. By placing its nose through the opening in the tube, the rat could sample the air delivered through the normally open port of the final valve [9]. A perforated glass disc [22] embedded in the base of the odour sampling tube diffused the incoming air stream. A flexible plastic hose [23] connected the top of the odour sampling port to an exhaust fan, located in the ceiling of the test room. This exhaust fan had a power rating of 100 cubic feet per minute.

The light source (General Electric # 1820) [19] and the photocell (Model LT 536 AB) [20] were encased in Arborite blocks and positioned across the opening of the odour sampling tube. The Arborite blocks were secured by metal clips to either side of the circular opening in the odour sampling port so that a "nose poke" by the subject into the sampling port broke the photobeam.

Water was delivered to the subject via an 18 gauge stainless steel drinking tube [21] located next to the odour sampling port. The drinking tube was connected to the grid of the floor [17] to form a touch sensitive circuit. Consequently, the animal's licking response completed the circuit and initiated delivery of water from a reservoir constructed from a 10 ml Becton Dickinson syringe and controlled by a solenoid valve (GV Series 3).

6.2.5. The Power Supply

The stimulus valves [13 & 16], the final valve [9], the water valve and the photocell unit were operated by a 28 volt power supply (GSC Model No E1100DA) and the timing of opening and closing of these valves was controlled by an Apple IIe computer connected to the olfactometer by a digital interface built by Gordon Troop, Department of Psychology, Dalhousie University.

The stimulus valves, the final valve and the water valve received input from the Apple IIe computer through the Troop interface. A photocell amplifier and the drinkometer circuit

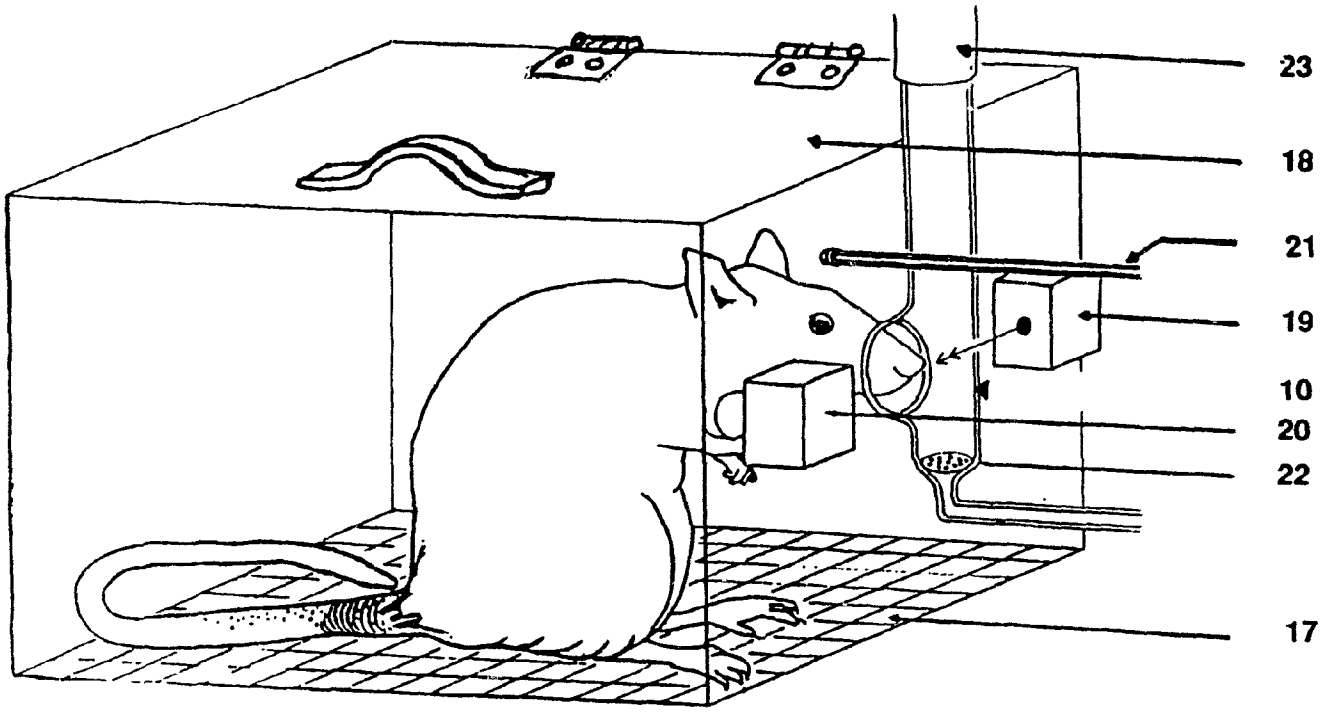


Figure 6.5. Details of the animal test chamber. (Numbers follow in sequence after those of Figure 6.3.)17. Grid floor; 18. Removable top; 19. Light source; 20. Photocell; 21. Drinking tube; 22. Perforated glass disk; 23. Exhaust hose.

provided input to the Apple IIe computer via the Troop digital interface. The photocell amplifier, the drinkometer circuit and interface for the second olfactometer were built by Gordon Troop.

The software required to control the olfactometer and record the rats' responses, was provided by Dr. Burton M. Slotnick (Psychology Department, American University, Washington, D.C.).

6.2.6. Olfactometer maintenance

To eliminate all residual urine odours, the olfactometer was cleaned after each discrimination task was completed, using the procedure described in Appendix I. To ensure that the air flow was the same in all of the stimulus channels, the flowmeters were calibrated using a bubble flowmeter after each cleaning. Construction and calibration of the bubble flowmeter is described in Appendix II.

6.2.7. Procedures for training rats in the olfactometer

6.2.7.(a) Routine procedures:

Prior to placing the animals in the test chamber each day, a number of routine procedures were carried out. These involved cleaning the test chamber, preparation of the odour stimuli and preparing the animal for testing.

1. Cleaning the test chamber. Prior to the first session of the day, the walls and floor of the test chamber, the sampling port and the drinking spout were wiped clean with 95 per cent ethyl alcohol.

2. Preparation of the odour stimuli. The vials of urine to be used as odour stimuli were thawed in a beaker of warm water and 1 ml of each sample applied to the cotton ball

in the labelled stimulus jar or placed directly in the custom made glass tubes via a syringe. The urines were discarded at the end of each day's testing. Stimulus jars were reused only for samples of the same individual donor's urine.

3. Preparing the animal for testing. Before being placed in the test chamber at the beginning of a session, each animal had its right hind paw rubbed with a solution of glycerin and salt. This ensured that proper contact was made with the grid floor to complete the drinking circuit.

6.2.7 (b) Preliminary training:

Training rats to discriminate odours involved a preliminary four-stage training procedure (Slotnick & Schoonover, 1984) in which the animals learned to obtain water reinforcement after breaking the photobeam and sampling the odour for a specific length of time. At any stage in the procedure, the session could be interrupted and the animal returned to the previous stage for further training if necessary.

Stage I: Orientat' on to the drinking tube. In a 200 trial orientation session, the subjects were trained to lick a drinking tube for a 0.02 ml water reinforcement. The number of licks required per reinforcement was increased from 1 to 20 over the first 20 trials of the session and for the remainder of the session, the number of licks required varied randomly from 1 to 30.

Stage II: Nose poke training. The 100 trial sessions in this stage of training began with 40 "refresher" trials in which the animal was required to lick the drinking tube a random number of times, varying from 1 to 30, for water reinforcement. The animal was then required to poke his nose into the odour sampling port far enough to break the photobeam prior to licking the drinking tube for reinforcement. When a nosepoke broke the photobeam, a stimulus channel solenoid valve operated and a stimulus odour was

presented. Urine collected from a male Long Evans outbred rat was used as the training stimulus.

The amount of time the animal was required to keep his nose in the sampling port was then gradually increased from 0.1 to 0.3 seconds over the remaining 60 trials in the session. No reinforcement was available if the rat failed to break the photobeam for the required length of time. Once a nosepoke occurred for the required length of time, the next trial could not be initiated until the licking response was made.

Stage III: Final valve delay training. The 200 trial sessions in this stage consisted of two phases. The first 80 trials were an extension of Stage II in that the time required to sample the odour after the photobeam was broken by a nosepoke increased from 0.16 to 0.25 seconds. Too short a sampling time resulted in a 4 second delay before another trial could be initiated. Even if the correct sequence of breaking the photobeam with a nosepoke and sampling the odour was completed, another trial could not be initiated until the animal received reinforcement by licking the drinking spout. Odour presentation lasted for a maximum of 2 seconds but terminated simultaneously with reinforcement when the animal responded correctly.

In the next phase, when the animal initiated a trial by breaking the photobeam, the final valve and the stimulus valve operated simultaneously so that the main airstream was diverted to exhaust. This ensured that the odour mixed completely with the main air prior to its entry to the test chamber.

The final valve closed for 0.2 seconds for the first 20 trials of this phase. Over the next 100 trials, the length of time the final valve operated gradually incremented so that in the last 20 trials, the animal was required to wait 0.7 seconds before the odour was introduced for sampling. If the animal consistently withdrew his nose from the odour port before the final valve had reopened, the previous time constraints were reintroduced until

the animal learned to wait for the final valve to reopen and the odour to be delivered. Odour discrimination training began as soon as Stage III was completed.

Stage IV: Urine odour versus no odour discrimination. The parameters in place at the end of Stage III were also used during this stage. The animal received water reinforcement only after he broke the photobeam with a nose poke, held his nose in the odour sampling port for 0.7 seconds while the main valve diverted air from the sampling port, sampled the odour for a minimum of 0.25 seconds and licked the drinking tube. The odour valve remained open for a maximum of 2 seconds. No reinforcement was available during a 2 second fixed interval immediately following the offset of the main valve and the presentation of the stimulus odour. In addition, a 4 second intertrial interval was introduced. Figure 6.6 outlines the sequence of events for an S+ trial in Stage IV.

Urine collected from the same outbred Long Evans male rat used in Stage III provided the positive stimulus odorant (S+) in Stage IV. Subjects were first presented with two blocks of 20 S+ trials; then, no-odour (clean air) trials were introduced as the negative stimulus trials (S-). The session continued with two hundred trials (100 S+ and 100 S-) presented in random order with the following three constraints: 1) the session began with an S+ trial; 2) an equal number of positive and negative trials occurred in each block of twenty trials; and 3) in any one block, no more than three S+ or S- trials were presented in succession. Two sessions of 240 trials were run with 40 introductory S+ trials given prior to the 200 S+ and S- trials.

The remainder of the sessions required to learn this odour/no odour discrimination omitted the blocks of S+ only trials and began with 100 S+ and 100 S- trials presented under the constraints listed above. Unless otherwise specified, when an animal scored a minimum of 85 per cent on the first block of 20 trials and a mean of 85 per cent or higher on each of the

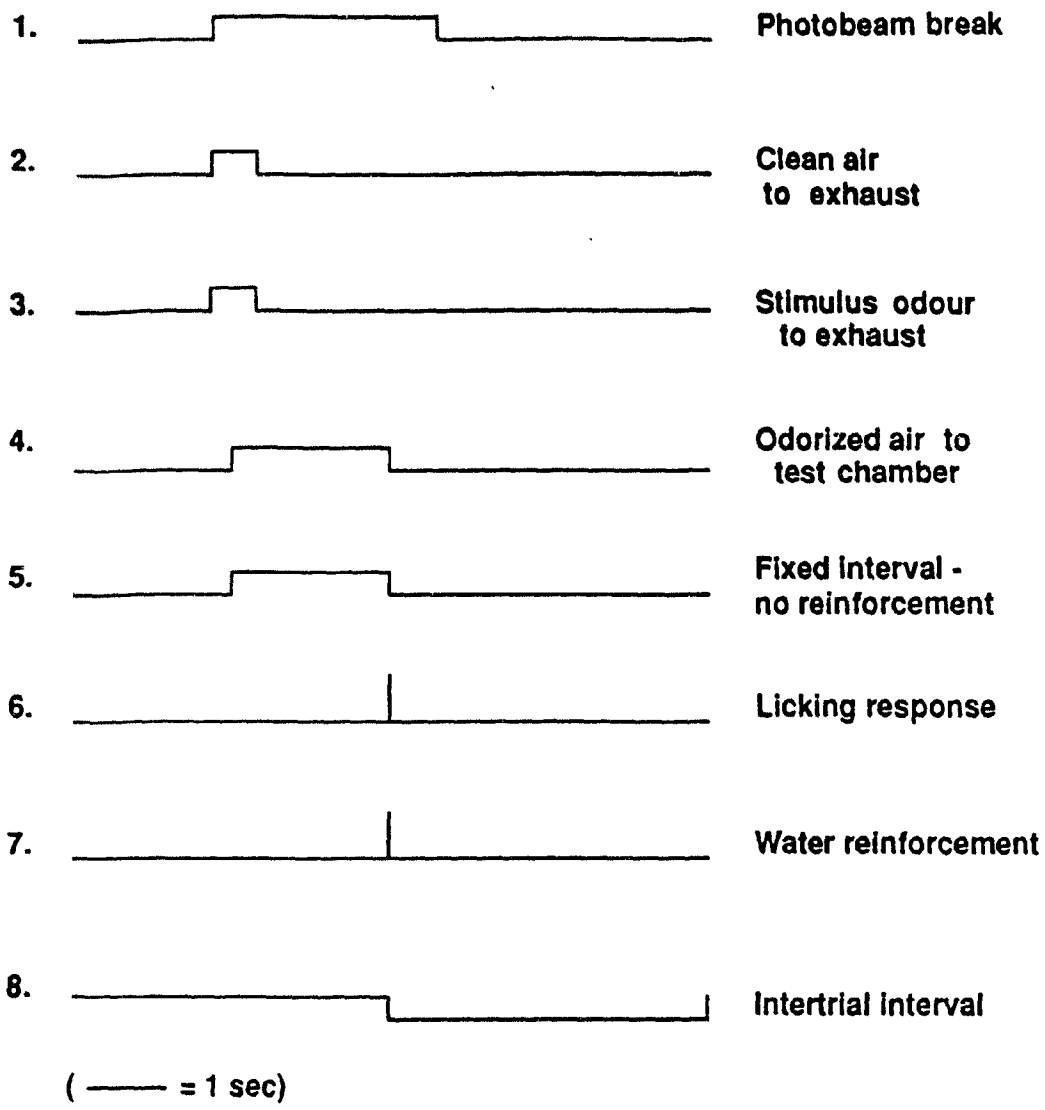


Figure 6.6 A diagram of the sequence of events on an S+ trial. 1. The subject initiates and maintains a photobeam break for a minimum of .95 sec. 2. The final valve diverts the airflow to exhaust for .7 sec. 3. The stimulus valve opens and an odour is diverted to exhaust for .7 sec. 4. The final valve operates and the odour is diverted to the sampling port for a max. of 2 sec. 5. A 2 sec fixed interval is in place when no reinforcement is available. 6. A licking response results in reinforcement. 7. A .02 ml water reinforcement is available for a correct response. 8. A 4 sec ITI follows the end of a trial.

remaining nine blocks of the 200 trial session, it was considered to have met the criterion for successfully acquiring the discrimination task.

6.2.7 (c) Odour Discrimination Tasks:

After successful completion of the odour versus no odour discrimination in Stage IV, the subjects progressed to the test sessions. As the procedure varied for each experiment, this information will be included separately within the appropriate chapters.

6.2.7 (d) Data Collection:

The data were recorded through input to the Apple IIe computer from the Bruce Field interface. Information collected for each block of 20 trials included (1) the per cent of correct responses; (2) the number of hits i.e., responses to the S+; (3) the number of misses i.e., S+ not responded to; (4) the number of correct rejections of S-; (5) the number of false alarms to S-; (6) the mean sampling time for the S+ and the S- (averaged over the 100 odour presentations for S+ and S-), and (7) the mean sampling time for both the S+ odour and the S- odour, i.e., the length of time the animal sampled each odour type averaged over the ten presentations.

Other information recorded for all 10 blocks in each session included (1) the number of short samples i.e., trials in which the animal did not sample the odour long enough; (2) the mean intertrial interval (ITI) in seconds for the 20 trials; (3) total errors; (4) total reinforcements obtained; (5) total length of session (in min); (6) time elapsed for each block (in sec) and (7) the total amount of water obtained. A sample data page is provided in Appendix III.

6.3. Hypotheses to be tested

1. Are the urine odours of individuality of germfree mice discriminated by rats? (Experiments 1 and 2)

Bacteria were found to be essential for the production of individual odours in rats (Singh et al., 1990) but not in mice (Yamazaki et al., 1990). Thus, the first two experiments in this thesis (Chapter 7) attempt to resolve these contradictory findings. These experiments examined the ability of rats to discriminate between urine from pairs of conventionally housed and germfree mice in the olfactometer (Experiment 1) and the habituation-dishabituation apparatus (Experiment 2). If bacteria are essential for the production of individual odours, pairs of conventionally housed mice should be discriminated but pairs of germfree mice should not. If bacteria are not essential, the odours of germfree mice should be discriminated as readily as those of conventionally housed mice.

2. Are the urine odours of individuality of rats removed by elimination of gram negative or gram positive gut bacteria? (Experiments 3 &4)

If bacteria are necessary for the production of individually unique urine odours, modification of these bacteria should alter the individual's odour. The second pair of experiments in this thesis (Chapter 8) assess whether the removal of gram negative or gram positive bacteria eliminates odours of individuality. Rats were tested in a habituation-dishabituation task for their ability to discriminate between the urine odours of two

different rats which have had these bacteria removed. If removal of different types of bacteria eliminates the odours of individuality, the odours of rats with bacteria removed should not be discriminable; if removal of specific bacteria only modifies odours of individuality, the odours of the same individual before and after the removal of gram negative or gram positive bacteria should be discriminable. These hypotheses were tested by using a habituation-dishabituation task to examine the responses of rats to different odours.

3. Will rats be able to detect changes in urine odours of individuality in the olfactometer after selective removal of bacteria? (Experiments 5 and 6)

To determine whether rats tested in the operant-olfactometer are able to discriminate urine odours of individuality after removal of bacteria, I presented probe trials of urine odours from the donors who had their gram negative or gram positive bacteria removed to subjects who have been previously trained to discriminate between the odours of the donors prior to the selective removal of their bacteria (Chapter 9). If subjects respond to the probe odours as S+ odours, then removal of bacteria has had no effect on the odours. A non-response to a probe odour indicates that removal of bacteria has altered the odour.

In these experiments, I also investigated whether a non-response to a probe odour means that the odour is completely different or somewhat different from the training odour. This was done by comparing the rate at which rats learned to discriminate between samples from the probe odour donors versus the odours of completely unknown individuals. If the discrimination of the odours from the probe odour donors is learned at the same rate as discriminating unknown individuals, one could conclude that altering the bacteria has completely changed the odour of the individual. If the probe odours are more easily

discriminated than the odours from the u. known individuals, one could conclude that the probe odours contained some components which were familiar to the rats, i.e., they were similar to the training odours.

4. A e rats able to discriminate between the urine odours of genetically identical mice maintained on different diets? (Experiment 7)

Since some urinary components are formed by the action of gastrointestinal bacteria on dietary products (Peppercorn & Goldman, 1972), dietary changes might alter individual urinary odours independently of genetic differences. If the odours of genetically identical mice on different diets are discriminable, rats should be able to discriminate between their odours before and after their diets changed (Chapter 10).

5. Do dietary and gentic cues interact in the production of urine odours of individuality of MHC congenic mice which differ at one locus? (Experiment 8)

The MHC influences the urine odours of mice (Boyse et al., 1991) and the results of experiment 7 indicate that diet can also influence odours of individuality. Genetic and dietary factors may interact to produce a unique odour profile. To test this hypothesis, I examined the ability of rats to discriminate between the urine odours of two strains of MHC congenic mice maintained on two different diets (Chapter 11). If the odours of mice of different strains on different diets are easier to discriminate than the odours of genetically identical mice on different diets or MHC congenic mice on the same diet, this would indicate that both factors contribute to odours of individuality.

6. Is it easier to discriminate between the urine odours of MHC congenic mice which differ at three loci than at one locus? (Experiment 9)

It is possible that two mouse strains which differ at three MHC loci would show a more salient difference in urine odour than two strains which differ at only one locus. If the odours of mice which differ at three loci are easier to discriminate than the odours of mice which differ at three loci, the rats should make fewer errors in learning the discrimination. This hypothesis was tested in Experiment 9 (Chapter 11).

7. Will a diet which is known to alter gut bacteria eliminate urine odours of individuality of rats? (Experiment 10)

Leon (1974) stated that caecal bacteria are eliminated in individuals which are maintained on a diet which contains sucrose as its only carbohydrate. To determine if caecal bacteria alter or eliminate the odours of individuality, I tested the ability of rats in a habituation-dishabituation task to discriminate between the urine odours of rats before and after they had their caecal bacteria altered by a test diet (Chapter 12). If the odours of individuality are removed by the elimination of caecal bacteria, rats should not be able to discriminate between the odours of two individuals on the sucrose diet. If the removal of caecal bacteria modifies the odours of individuality, the odours of the same individual should be discriminable before and after changing to the test diet which eliminates the caecal bacteria.

8. Are rats able to learn the concept of MHC as a signal of individuality and will this concept be disrupted by a change in diet? (Experiment 11)

Yamazaki and his colleagues (Yamaguchi et al., 1981; Yamazaki et al., 1982) found that the MHC provided constant cues which were discriminable in the urine. To affirm that rats can learn the "MHC concept", I trained rats to discriminate between urine samples from MHC congenic conspecifics and assessed their ability to transfer their training within a session when they were given samples from novel individuals of the same strains on the same diet (Chapter 13). If the rats are able to learn the concept of MHC, they should transfer this learning and continue to discriminate between the odours of novel individuals of the same MHC types without showing a decrement in performance.
To further determine the role of diet in influencing cues of individuality, I evaluated the ability of these rats to generalize their learning of MHC congenic strains to the odours of these same individuals after their diet had been changed. If the performance of the rats is disrupted when the diet of the odour donor rats is changed, this would provide evidence that cues provided by diet influence a discrimination based upon genetic differences.

9. Will the ability of rats to discriminate between the urine odours of genetically identical rats on different diets be disrupted by changing the strain of rats but not their diet? (Experiment 12)

Using the transfer of training paradigm established in Experiment 11, I examined whether the ability of rats to discriminate between the urine odours of genetically identical rats on two different diets is disrupted when the urine odours are switched to different

strains of rats on the same diets (Chapter 13). In this way, the relative influence of dietary and MHC cues could be assessed. If the performance of the rats is disrupted when the strain of the odour donor rats is changed, this would provide evidence that cues provided by the MHC influence the discriminability of odours.

6.4. Presentation of the results

The results of the habituation-dishabituation and operant-olfactometer experiments are presented in the following seven chapters. In some instances, to provide the appropriate context, a chapter will begin with a short introduction which summarizes the literature most relevant to the research question. This literature will have been fully reviewed in Chapters 4 and 5.

The methods section will include information regarding the subjects and urine donors as well as any procedural details not found in Chapter 6. A comprehensive results section will follow. Each chapter will close with a discussion of the findings.

Chapter 14 of this thesis will summarize the results of each experiment and also present a model which illustrates the contribution of genetic and environmental factors to the overall odour profile of an individual.

CHAPTER 7

ARE THE URINE ODOURS OF INDIVIDUALITY OF GERMFREE MICE DISCRIMINATED BY RATS?

EXPERIMENT 1

ARE THE URINE ODOURS OF GERMFREE MICE DISCRIMINATED BY RATS IN AN OPERANT-OLFACTOMETER?

It has been well established that the MHC provides the genetic basis for an individuality marker in the urine of rats and mice (Brown, Singh, & Roser, 1987; Brown, Roser & Singh, 1989; Boyse, Beauchamp, Yamazaki, & Bard, 1991). Recent findings suggest that commensal bacteria are also involved in the production of individually distinct urine odours in rats. Using a habituation-dishabituation task, Singh et al. (1990) determined that rats could not discriminate between the urine of germfree MHC congenic rats (PVG vs PVG.R1). When donors were moved to conventional housing, their urines could be discriminated. Also, rats trained in a go-no go operant task in an olfactometer made more errors in learning to discriminate between urines of germfree MHC congenic rats than between urines of conventionally housed MHC congenic rats (Schellinck, Brown, & Slotnick, 1991). Although the rats were able to discriminate between urine samples from individual germfree MHC congenic rats, they consistently performed at chance at the beginning of each session. This inability to retain the information from session to session indicates that constant cues were not being used to discriminate between the urine samples from germfree rats. Moreover, when the urine samples from germfree rats were changed

to others of the same strain in mid-session, performance was also disrupted. Urine odours from conventionally housed MHC congenic rats were readily discriminated; the discrimination was remembered from session to session and no drop in performance was observed when the samples were changed to different donors of the same MHC type in mid-session.

The finding that germfree rearing eliminates odours of individuality in rats is consistent with the two hypotheses described in Chapter 3 which suggest an interaction between the MHC and bacteria in producing odours of individuality: the "Howard hypothesis" which postulates that the MHC determines which commensal bacteria are present in the body so that each individual has a different population of bacteria and hence a unique pattern of urinary volatiles (Howard, 1977) and the "carrier hypothesis" which suggests that the unique urine odour arises from the volatiles produced by a pool of commensal bacteria which are specifically selected for binding in the pocket of individually unique MHC glycoproteins (Pearse-Pratt et al., 1992).

Regardless of the theoretical constructs which create a place for bacteria in the production of odours of individuality, the fact is that the urine odours of germfree MHC congenic mice appear to be discriminated as readily as those of conventionally housed animals by mice which were trained in a Y-maze (Yamazaki et al., 1990). The test mice could also transfer their learning from urine samples of conventionally housed mice to samples from germfree mice. Such a finding would suggest that, in mice, the germfree status of the urine donors does not influence the MHC determined odour type. To determine if rats could also detect consistent cues in the urine odours of germfree mice, I examined the ability of rats to discriminate between the urine odours of conventionally housed and germfree B6 and B6-H-2^k mice in the operant-olfactometer.

I used rats to examine the discriminablity of urine odors of mice because I am interested in knowing if differences exist between certain odors and if some differences are easier to remember than others. It has been shown that rats can discriminate between such similar odours as ethyl acetate and isopropyl acetate (Slotnick & Risser, 1990) and between different concentrations of the same odor (Slotnick & Ptak, 1977). Regardless of whether the odors are produced by mice or some other species, if the genetic difference between these strains of mice manifests itself as a difference in odor, rats should be able to detect this. Also, if a change in diet affects the discriminablilty of urine, the rats should also be able to determine this.

METHOD

Subjects

Six male Long Evans rats were purchased from Charles River Canada Ltd. (St Constant, Que.) at 90 days of age and housed individually in 48 x 27 x 16 cm polycarbonate cages with stainless steel grid tops and softwood chip bedding. The animals were kept on a reversed 12:12 light:dark (L:D) cycle with the lights off from 8 a.m. to 8 p.m. and were provided with Purina rat chow and water ad lib. Two weeks prior to training in the operant-olfactometer, water availability was restricted to 10-15 ml per day at 6 p.m. Prior to training for this experiment, these rats were subjects in an experiment in which they had learned to discriminate between the urine odours of outbred and MHC congenic rats.

Urine Donors

The urine donors were male B6 and B6-H-2^k mice, two MHC congenic strains which differ at 3 loci of the MHC (Yamaguchi et al., 1981). The urine samples were provided by Dr. Gary Beauchamp and Dr. Kunio Yamazaki from the Monell Chemical Senses Center, Philadelphia. The urine from the conventionally housed B6 and B6-H-2^k donors was collected at Monell. The urine from the germfree B6 and B6-H-2^k donors was supplied to Monell by Taconic Farms, Germantown, New York. To ensure that the mice were germfree, individuals were tested weekly at Taconic Farms for the presence of bacteria. In addition, urine samples were cultured for mold and bacteria at Monell and found to be negative (Yamazaki et al., 1990). Prior to being packed in dry ice and shipped from Philadelphia to Dalhousie University, the samples were stored at -20^o C. The samples were kept frozen at -20^o C at Dalhousie University until needed.

Apparatus and Procedure

Rats were tested in the olfactometer described in section 6.2. The six subjects were required to discriminate between the following pairs of odours: (1) pooled urine from conventionally housed B6-H-2^k and B6 mice (Task 1) and (2) pooled urine from germfree B6-H-2^k and B6 mice (Task 2). One strain was designated the S+ for three of the subjects and the other strain was the S+ for the other three subjects. Three of the subjects were tested with the urines of conventionally housed mice first while the other three subjects learned to discriminate between germfree mice first. Each subject was tested for eight sessions (1600 trials) on each task.

At the midpoint of each session (after 100 trials were run), a second pair of S+ and S- samples from the same strains were introduced to determine if the subject's performance was influenced by individual odour cues even though the urine donors were genetically identical to the donors in the first 100 trials.

Statistical Analysis

A repeated measures ANOVA was used to examine the differences in learning the two tasks. Newman-Keuls post hoc comparisons were used to determine which means differed from each other. The analysis of the number of errors on each task was based upon the errors on the first 5 blocks of trials because of the introduction of different samples on block 6 of each session.

RESULTS

Based upon the criterion of a score of 80 % correct on the first block of trials and a mean of 80 % on the remaining blocks, five of the six rats learned to discriminate between the urine odours of B6-H-2^k and B6 congenic mice raised in conventional housing and under germfree conditions. Of these five rats, three (Magnus, Trinity, & Winchester) learned and remembered the cues for the discrimination between the urine odours from conventionally housed B6-H-2^k and B6 mice for three consecutive sessions. Three rats (Howard, Magnus, & Trinity) also remembered the discrimination between the urine odours of B6-H-2^k and B6 mice raised in germfree conditions for three consecutive sessions. The learning curves for one rat are shown in Figure 7.1. The other subjects were able to meet criterion in both tasks for at least one of the eight sessions.

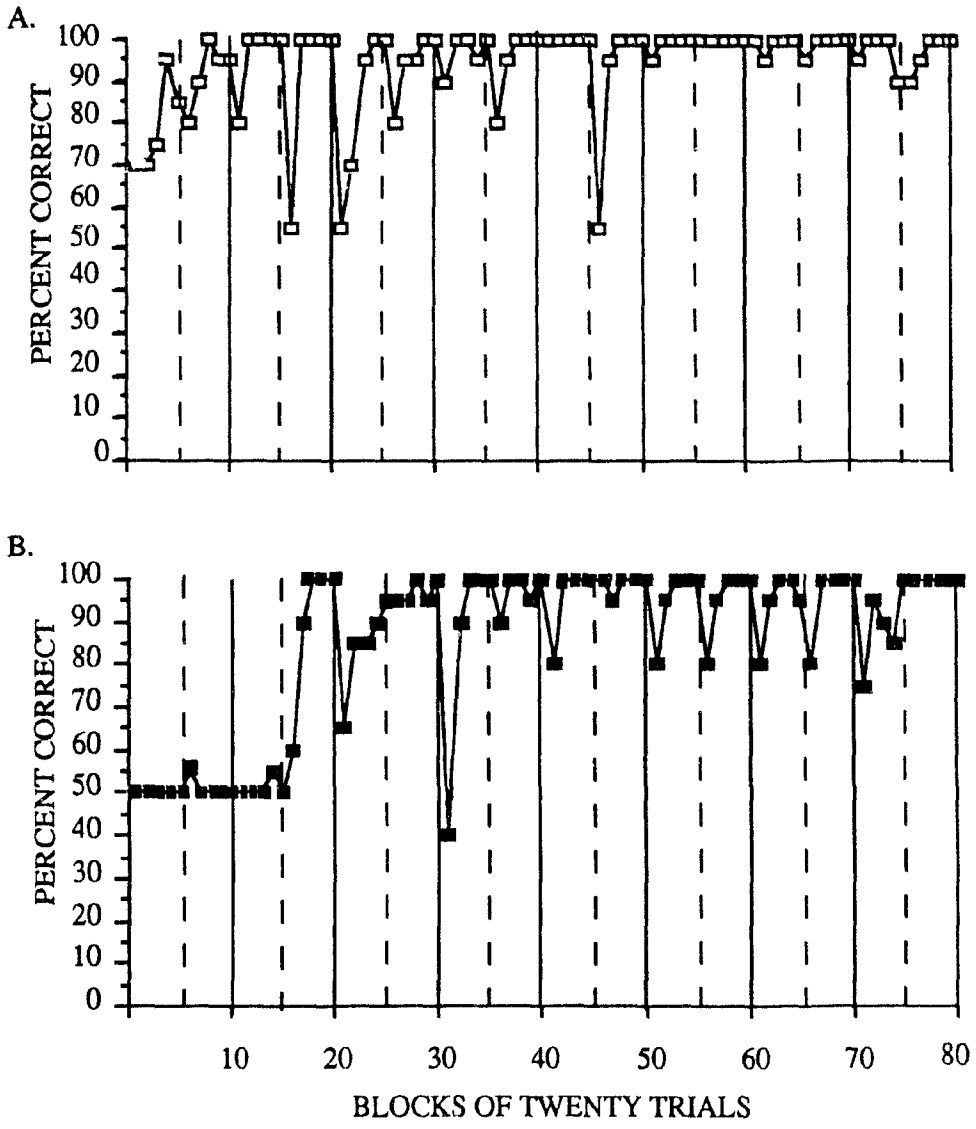


Figure 7.1. The learning curves of one rat (Trinity) for eight sessions in the discrimination of urine odours from A. MHC congenic mice raised in conventional housing and B. MHC congenic mice raised in germfree conditions. On block five of each session, the stimuli were replaced with different samples from different individuals of the same strains.

Analysis of the mean number of errors to criterion on the first five blocks of trials of each session indicated that the two tasks did not differ significantly with respect to degree of difficulty [$F(1, 5) = .219$, $p>.05$] but that fewer errors were made on both tasks as the amount of training increased [$F(7, 35) = 10.4$, $p<.001$]. Newman-Keuls post hoc tests showed that the number of errors was significantly lower in sessions four to eight than on session one. The errors in sessions one to three did not differ (Figure 7.2).

The introduction of different samples of test odours on block 6 of each session did not result in a significant change in the number of errors between blocks 5 and 6 in the two tasks. A repeated measures ANOVA indicated that there was no significant main effect of odour type [$F(1,5) <1.0$] or session [$F(7,35) = 1.04$] nor a significant interaction (Table 7.1). An examination of the range of difference scores between blocks 5 and 6 for the two tasks across sessions reveals that the rats' performance showed little disruption with the introduction of different samples on block 6 with the errors on Task 1 ranging from 0.83 - 3.5 and those on Task 2 ranging from 1- 3.67.

DISCUSSION

These results indicate that the urine odours from germfree MHC congenic mice can be discriminated by rats in an operant olfactometer. The results of this experiment are similar to those of Yamazaki et al. (1990) which showed that the odours of germfree mice can be discriminated by mice in a Y-maze. We are, thus, left with the question as to why the urine of germfree MHC congenic mice can be discriminated while the urine of germfree MHC congenic rats cannot. If, as Yamazaki et al. (1990) conclude, germfree status is irrelevant to the production of individual odours, then what caused the odours of individuality to be removed from the germfree rat samples used by Singh et al. (1990)?

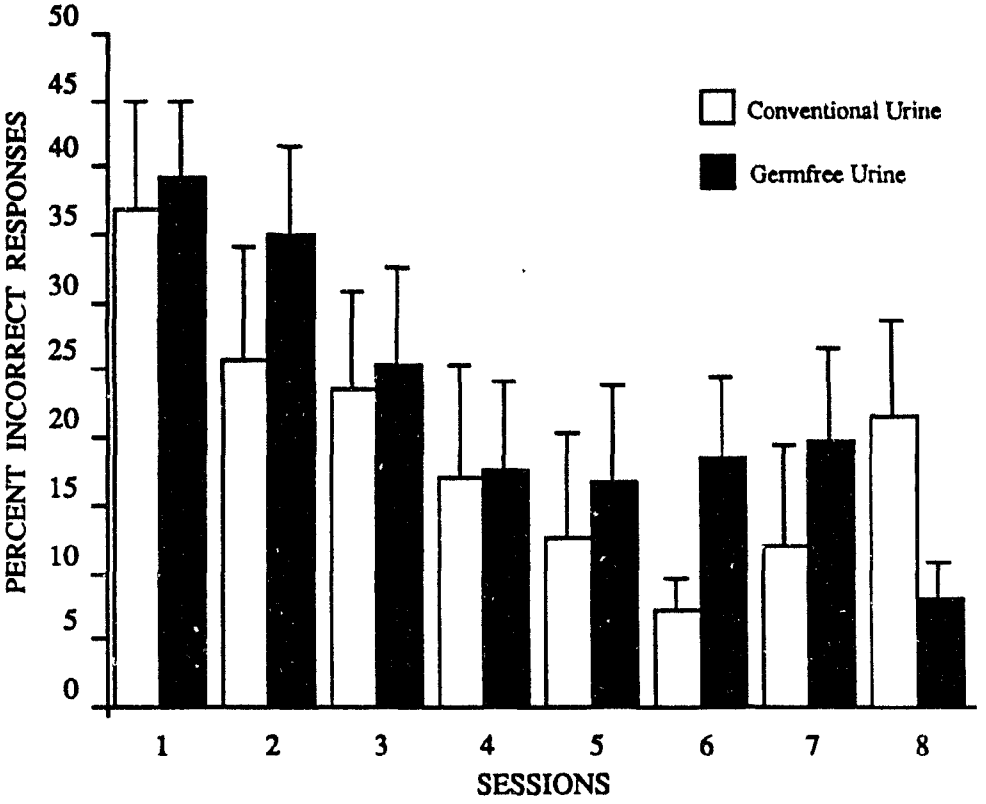


Figure 7.2. The mean percent of incorrect responses (± sem) over eight sessions in the discrimination of urine odours from conventionally housed and germfree reared mice. The analysis is based upon the number of errrors in the first 100 trials of each 200 trial session.

Table 7.1. The change in the mean (± sem) number of errors between blocks 5 and 6 in two urine odour discrimination tasks for eight successive sessions: (1) the discrimination of conventionally housed MHC congenic mice; (2) the discrimination of germfree raised MHC congenic mice.

SESSION	CONVENTIONAL	GERMFREE
1	1.83 ± .41	1.00 ± .63
2	3.50 ± 1.23	1.67 ± .67
3	2.17 ± .79	3.67 ± 1.69
4	1.83 ± 1.04	1.00 ± .45
5	3.17 ± 1.37	1.67 ± .80
6	2.17 ± 1.25	1.67 ± .76
7	.83 ± .31	1.33 ± .42
8	1.83 ± .70	2.33 ± .84

Conversely, if bacteria do contribute to the production of an individually unique urine odour, what other variables created the discriminable differences found in the germfree mouse urine in this experiment and in the investigation by Yamazaki et al. (1990)? There are several methodological factors which might account for the discrepancy between the findings of the rat and mouse studies. First, there may be differences in the urine samples, themselves. Both labs confirmed the germfree status of their urine donors; however, the samples differed in a number of other respects. The mouse urine donors used by Yamazaki et al.(1990) differed at three MHC loci whereas the rat urine donors used by Singh et al. (1990) and Schellinck et al. (1991) differed only at the class Ia locus. As noted in Section 3.2, the H-2 complex and its closely linked genes may influence quantitative metabolic variations (Ivanyi, 1978; Klein, 1986). Whether the MHC in rats produces such changes is unknown, nor is it known whether differences at three MHC loci rather than one would differentially influence the discriminability of the odour cues in the urine.

Second, the urine samples used by Singh et al. (1990) and Schellinck et al. (1991) were from individual rats; the samples used by Yamazaki et al. (1990) were pooled from a number of individual mice. While the use of pools would eliminate spurious differences within a strain in the urine, it would compound the effect if there are actual differences between strains which are expressed in the urine but which are unrelated to cues of individuality. If the absence of bacteria eliminates the most salient cues, it is possible that these less relevant cues would be sufficient to produce constant discriminable differences in the pooled mouse urines.

Third, the diet supplied to the germfree rat urine donors used by Singh et al. (1990) was irradiated; the diet given to the germfree mice was sterilized (Beauchamp, 1991, personal communication). As sterilization alters the protein quality and changes the bile

acid composition of the diet and irradiation does not (Coates, 1984a; Wostmann, Beaver, Chang & Madsen, 1977) metabolic products may have been excreted in the urine of the germfree mice which would not be present in the urines of the germfree rats.

Fourth, there may be extraneous differences in the urines. Because of the absence of bacteria in germfree animals, macromolecules are present which cause impaired water absorption in the gut; also, there are no bacterial enzymes to degrade mucus secreted by the intestine. Consequently, animals kept in germfree conditions have more liquid contents in the cecum and colon and are subject to chronic diarrhea (Van der Waaij, 1984). This makes it difficult to collect urine samples which are free from fecal matter. In order for this to influence the discriminability of the germfree mouse urines, the gut of the two different strains of mice would have to be differentially affected by their germfree status, thus producing consistently discriminable differences in the urines of the two different strains. In fact, the samples used in this experiment did contain extraneous substances and the urine from one strain of mice was darker in colour than the other. Whether this difference in colour was caused by contaminants or was indigenous to the urine, itself, was not determined. As the constituents of the urine samples were not identified, the source of these differences remains a matter of speculation.

Despite the possible differences between rat and mouse urine donors and the urine samples collected, it may be that bacteria are actually necessary for the production of individually unique urine odours in rats and not in mice and that MHC differences in mice can be expressed without bacterial influence. In rats, when the MHC class I antigens are removed, the urine odours are still discriminable (Brown, Roser and Singh, 1989) whereas the effect of removing the class I antigens in mi. . is unknown. If investigation reveals that removing these antigens from mouse urine eliminates the discriminability of the samples,

this would suggest that different mechanisms are responsible for production of odours of individuality in rats and mice.

Nonetheless, it should also be noted that the experimental procedures and criteria used to assess the discriminability of the urine odours of germfree rats and mice may not be comparable. Three different procedures have been used to examine the discriminability of germfree urines: (1) rats were tested in a habituation-dishabituation paradigm (Singh et al., 1990); (2) they were also trained in a go/no go operant olfactometer task (Schellinck et al., 1991) as well as in this experiment. (3)Yamazaki et al. (1990) used a Y-maze for their studies with mice. While the latter two instrumental conditioning procedures are comparable, they have both introduced an artificial component into the discrimination process. The subjects were water deprived and received water reinforcement for discriminating between the samples. Thus, they were highly motivated and may have detected differences between urine odours that they would not normally attend to. To verify these results, the same experiment was done in the habituation-dishabituation apparatus.

EXPERIMENT 2

ARE THE URINE ODOURS OF GERMFREE MICE DISCRIMINATED BY RATS IN A HABITUATION-DISHABITUATION TASK?

The habituation-dishabituation task examines the rats' ability to detect odour differences in a more natural situation, and may be a more reliable indicator of the animal's discrimination processes. In Experiment 1, rats were able to discriminate between the urine odours of germfree mice in an operant task. To determine whether procedural differences

could have influenced the outcome of the experiments, in this experiment, the ability of rats to discriminate between the urines from germfree mice in a habituation-dishabituation paradigm was assessed.

METHOD

Subjects

Thirty-two male Sprague Dawley rats purchased from Charles River Canada, St. Constant, Quebec at 90 days of age were used as test subjects. They were individually housed in 25 x 19.5 x 18.5 cm Wahman hanging cages, provided with Purina Lab Chow and water ad libitum and maintained on a 12:12 reversed light:dark cycle, with lights off at 8:00 a.m.

Urine Donors

The urine donors were the same conventionally housed and germfree male B6 and B6-H-2^k donors described in Experiment 1.

Apparatus and Procedure

The habituation-dishabituation apparatus described in section 6.1 was used in this experiment. Each subject received seven sequential two minute odour presentations. Water was presented for the first three trials, followed by three presentations of urine sample A and, because of a shortage of urine, only one presentation of urine sample B was

given. For each two minute trial, the time spent rearing and sniffing each odour stimulus was recorded with a stopwatch. Two experimenters ran each test: one placed the urine samples on the filter paper and the second, who was blind as to which urine odour was present, scored the sniffing responses of the rats.

The subjects were tested with urine samples from one of four different pairs of mice:

A) conventionally housed male mice of the two MHC congenic strains, B6 and B6-H-2^k (8 subjects tested);

B) conventionally housed male mice of the same strain (4 subjects tested with urine from B6 mice and 4 subjects tested with urine from B6-H-2^k mice;

C) germfree male mice of the B6 and B6-H-2^k strains (8 subjects tested);

D) germfree male mice of the same strain (4 subjects tested with urine from B6 mice and 4 subjects tested with urine from B6-H-2^k mice).

In the first two tests, the presentation of urine samples was counterbalanced, so that half of the subjects in each test received the odours of B6 mice first, while the other half received the odours of B6-H-2^k mice first. All subjects were tested during the dark phase of the light:dark cycle, under dim white illumination.

Statistical Analysis

A randomized block ANOVA was used to examine differences in the amount of time spent sniffing the odour samples over the seven trials. Newman-Keuls post hoc comparisons were used to determine which means differed from each other.

RESULTS

Rats discriminated between the urine odours of conventionally housed MHC congenic mice of the B6 and B6-H-2^k strains (Figure 7.3A). There were significant differences in the investigation times over the seven odour presentations [$F(6, 42) = 14.82$, $p < 0.001$] with more time spent investigating the urine odour samples on trials four and seven than on any other trial ($p < 0.05$). Thus, the subjects showed a dishabituation of the odour investigation response when presented with the first urine trial (trial 4) and when first presented with a urine sample from a different strain (trial 7).

In contrast, the subjects did not discriminate between the urine odours of germfree MHC congenic mice of the B6 and B6-H-2^k strains (Figure 7.3C). There were significant differences in investigation time over the seven trials [$F(6, 42) = 7.61$, $p < 0.001$], but only trial 4 differed significantly from any other trial ($p < 0.05$). Thus, when subjects were presented with a novel urine sample from a germfree mouse of the second strain (trial 7), they did not dishabituate, indicating that they did not discriminate the second urine odour from the first.

Subjects were unable to discriminate between the urine odours of genetically identical individuals whether conventionally housed (Figure 7.3B) or germfree(Figure 7.3D). There were significant differences among the seven trials when the samples were from conventionally housed individuals of the same strain [$F(6, 42) = 19.64$. $p < .001$] and when they were from individuals of the same strain raised in germfree conditions [$F(6, 42) = 5.93$, $p < 0.001$] but post hoc comparisons showed that only trial 4 differed significantly from any other trial in each test, indicating that the rats did not discriminate between the urine odours of genetically identical mice, regardless of their bacterial status.

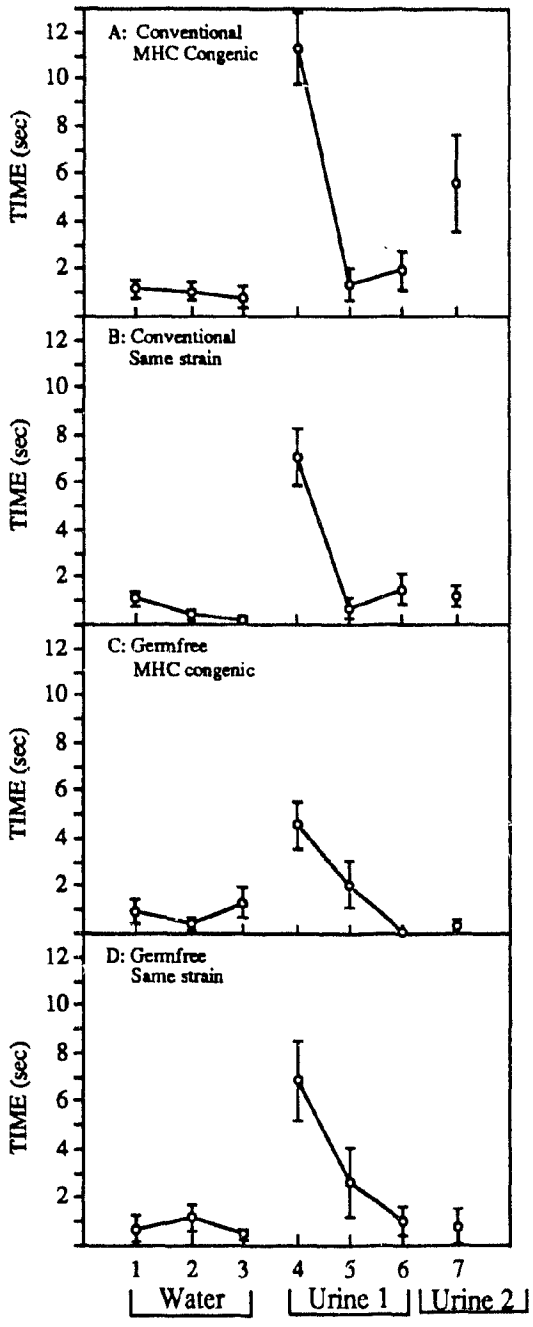


Figure 7.3. Mean (± sem) time (sec) spent rearing and sniffing urine odours from (A) conventionally housed MHC congenic mice; (B) conventionally housed genetically identical mice; (C) MHC congenic mice reared in germfree conditions and (D) genetically identical mice reared in germfree conditions.

DISCUSSION

Rats tested in the habituation-dishabituation paradigm did not discriminate between the odours of mice raised in germfree conditions. These results suggest that the presence of bacteria is necessary for the production of individually unique odours in mice. It has been previously determined that the urine odours of MHC congenic rats reared in a germfree environment were not discriminable by other rats in a habituation-dishabituation task (Singh et al., 1990), nor were they consistently discriminated by rats in an operant-olfactometer task (Schellinck et al., 1991). Thus, based upon these findings, one might conclude that bacteria play a role in creating discriminable differences in the urine odours of mice and rats.

Nonetheless, the results of this experiment conflict with the results of Yamazaki et al. (1990) and the findings from the previous study (Experiment 1) which showed that the urine odours of germfree mice were discriminated by rats in an operant conditioning task. What factors might account for the discrepancy between these results and those of the present experiment? Rats were highly motivated to perform in the operant olfactometer task because they were water-restricted and received a water reward for a correct discrimination. Rats used in the present experiment were not water-restricted. Moreover, in the operant olfactometer task, the rats were presented with 100 trials of each urine sample in one session. Similarly, Yamazaki et al. (1990) trained their experimental mice for up to 200 trials. Consequently, as a session progressed, the rats and mice may have learned to detect differences between urine samples in the operant task which would go undetected during the two minute sniffing trials of the habituation-dishabituation task.

Quantitative differences in hormone metabolites could have provided such discriminable cues. As noted in section 3.2, MHC congenic mice may vary in terms of

their testosterone or corticosteroid levels (Ivanyi, 1978). The changes in physiological activity which are influenced by such traits could subsequently be expressed as quantitative discriminable differences in urine metabolites. Although the presence of testosterone is not necessary for individual rats to be discriminated by their urine odours (Brown, 1988), urine odours of both rats (Taylor et al., 1982) and mice (Monahan et al., 1993) with different testosterone levels can be discriminated in a preference test. Also, corticosteroids are known to have significant effects on certain urinary chemosignals in mice (Drickamer & McIntosh, 1980). Thus, in learning to discriminate between the urine samples from germfree mice in an operant conditioning task, the rats may have been using cues provided by stable, quantitative differences when other cues of individuality were not available.

Another way to examine the discriminability of MHC congenic mice is to use mate choice experiments (Yamazaki et al., 1976; Eklund, Egid & Brown, 1991; 1992). One could also assess the ability of MHC-based odour differences in male mice to cause pregnancy block in females (Yamazaki et al., 1983). Using either of these procedures to test the discriminability of germfree MHC congenic mice may provide more conclusive evidence about the role of commensal bacteria in the production of an individual's unique odour.

It is becoming increasingly evident that genes other than those of the MHC (Beauchamp et al., 1990; Eggert et al., in press) and environmental factors such as diet (Brown & Schellinck, 1992) contribute to an odour profile. A number of methods can be used to assess the role of bacteria in producing odours of individuality. One of these is to examine the discriminablity of urine odours from germfree mice which differ in genes other than those of the MHC. This requires access to a germfree rearing lab which is not available at Dalhousie University.

Other ways to assess the role of bacteria in the production of odours of individuality are to alter the bacterial populations by drug treatment or to modify the dietary substrate upon which the bacteria act. In Experiments 3 to 6, I will use drugs to alter the bacterial populations; in Experiments 7 to 12, I will manipulate the diet of rats and mice to alter their bacteria.

CHAPTER 8

ARE THE URINE ODOURS OF INDIVIDUALITY OF RATS REMOVED BY ELIMINATION OF GRAM NEGATIVE OR GRAM POSITIVE BACTERIA?

Commensal bacteria appear to play a role in producing odours of individuality in rats (Singh et al., 1990; Schellinck et al., 1991) but the role of bacteria in producing unique odours in mice is less certain (Experiments 1 and 2). The urine of germfree rats differs from that of conventionally housed rats in a number of volatile components (Holland et al., 1983) and some of these volatiles may provide the basis of individual odours. As was described in Chapter 5, volatile as well as nonvolatile compounds are produced when foodstuffs are metabolized by the action of gut bacteria. Whether specific gut bacteria are responsible for the production of these volatiles is unknown.

While hundreds of species of gut bacteria exist, they, like all bacteria, may be divided into two groups, gram negative and gram positive, based upon their reaction in the gram staining process. Gram negative bacteria appear as blue-purple; gram positive bacteria appear red or orange (Alcamo, 1994). Gram negative bacteria include enteric gram negative rods such as *Escherichia coli*; gram positive bacteria include regular, nonsporing rods such as *Lactobacillus* and cocci such as *Streptococcus homionus* and *Streptococcus bovis*.

The latter bacteria were used by Singh et al. (1990) to colonize the gut of germfree rats which were introduced into a specific pathogen free colony. After inoculation with this cocktail, the odours of the rats were discriminable. Thus, it is possible that some combination of these bacteria were responsible for odours of individuality. As no analysis was made of the gut bacteria, however, the possibility that other bacteria present in the

colony room were also involved in this process cannot be eliminated. Nonetheless, any of these bacteria would also fall into the categories of gram negative or gram positive.

EXPERIMENT 3

THE EFFECT OF THE REMOVAL OF GRAM NEGATIVE GUT BACTERIA ON THE DISCRIMINATION OF URINE ODOURS OF RATS IN A HABITUATION -DISHABITUATION TASK

If the urine odour of a rat changes when specific groups of bacteria are eliminated and this change is detected by other rats, it can be concluded that removal of bacteria alters individual odours. If the odours of two rats cannot be discriminated following elimination of bacteria, it can be concluded that bacteria are necessary for the production of odours of individuality. To determine if specific classes of bacteria are involved in the production of odours, I tested rats in a habituation-dishabituation task for their ability to discriminate between urine odours of rats which had their gram negative bacteria removed by treatment with antibiotics.

METHOD

Subjects

Thirty-one male Sprague Dawley rats were used as subjects. They were individually housed in 25 x 19.5 x 18.5 cm Wahman hanging cages, provided with ad lib Purina Lab Chow and water and maintained on a 12:12 reversed L:D cycle, with lights off at 9:00 a.m.

Urine Donors

Urine was collected from six month old male Long Evans rats (n = 8) which had free access to Purina rat chow and water except during the eight hour urine collection period when only water was available. Urine was collected from the same rats before and after the removal of bacteria by placing the urine donors in a metabolism cage for eight hours per day during the dark phase of the L:D cycle.

Antibiotic Treatment

The drug regime necessary to remove the gram negative bacteria was provided by Dr. W. Hofstra, Laboratory for Medical Microbiology, University of Groningen, The Netherlands. The rats were given Novotrimel (Trimethoprim 80 mg and Sulfamethoxazole 400 mg) tablets in a concentration of 1.5 gm/L drinking water for two weeks to eliminate gram negative bacteria. Colistine sulfate (1 gm/L drinking water) was given for the first three days of the decontamination procedure. For these two weeks, the following procedures were followed: Cages of the rats were changed three times a week and with each cage change, they received new food and clean bottles with fresh antibiotics in their drinking water. After these two weeks, the cage changes and fresh food and water containing antibiotics were provided once weekly.

Bacteria Counts

Bacteria counts were taken before and after antibiotic treatment. One fecal bolus was collected from each urine donor and a colony count of gram negative bacteria was

determined at the Dept. of Microbiology, Victoria General Hospital, Halifax, Nova Scotia by the following method: the stool was emulsifed in an equal volume of sterile buffered saline and doubling dilutions performed with the assumption that the initial concentration is of the order of 10^{10} organisms per ml. These dilutions were then plated on MacConkey agar and colony counts performed from that for lactose fermenting organism and non-lactose fermenting organisms.

After the rats were maintained on the antibiotics for two weeks, fecal samples were again collected and analyzed and the gram negative bacteria were found to be absent (Table 8.1). Subsequently, urine was collected from these subjects for a second time. At the end of each collection day, the samples were removed from the containers, labelled and stored in 2 ml aliquots at -20^{o} C until used.

Apparatus and Procedure

Water or urine (0.1 ml) was presented on a 5.5 cm circle of filter paper in the habituation-dishabituation apparatus as described in Section 6.1. Each subject received nine sequential two minute odour presentations with water presented for the first three trials, followed by three presentations of Urine Sample A and three presentations of Urine Sample B. The time spent rearing and sniffing each odour was recorded with a stopwatch by an observer blind to the conditions. All subjects were tested during the dark phase of the L:D cycle, under dim white illumination and the test chamber was cleaned with a 70 % ethyl alcohol solution between subjects.

Table 8.1. Gm negative bacteria count in feces of rats given Novatrimel and Colysistine Sulfate.

	Before	After
Rat SD1	1,580,000 colonies/ml 95% lactose negative	0
Rat SD2	2,620,000 colonies/ml 100 % lactose positive	0
Rat SD3	76,000 colonies/ml 100 % lactose positive	0
Rat SD4	150,000 colonies/ml 100 % lactose positive	0
Rat SD5	320,000 colonies/ml 95 % lactose positive	0
Rat SD6	340,000 colonies/ml 100 % lactose positive	0
Rat SD7	86,000 colonies/ml 95 % lactose negative	0
Rat SD8	8,000,000 colonies/ml 95 % lactose positive	0

Subjects were tested with one of four different pairs of urine samples:

A) two samples from the same rat collected on different days before its gram negative bacteria were removed or two samples from the same rat collected after its bacteria were eliminated (8 subjects tested);

B) urine odours of the same rat before and after its gram negative bacteria were eliminated (8 subjects tested);

C) urine odours from two different rats before their gram negative were eliminated (7 subjects tested);

D) urine odours from two different rats after their gram negative bacteria were eliminated (8 subjects tested).

Statistical Analysis

A randomized block ANOVA was used to examine differences in the amount of time spent sniffing the odour samples over the nine trials. Newman-Keuls post hoc comparisons were used to determine which means differed from each other.

RESULTS

In the first test, rats were presented with different samples from an individual rat prior to or following the removal of its gram negative bacteria (Figure 8.1A). A repeated measures ANOVA revealed significant differences in the investigation times over the nine odour presentations [$F(8, 56) = 14.97$, $p < 0.001$]. Post hoc testing indicated that only trial 4 differed significantly from any other trial ($p < 0.01$). Thus, subjects responded to the first urine sample after the water presentations on trial 4, but did not respond differently to a second urine sample from the same rat on trial 7 (Figure 8.1A). Thus, the two samples from the same rat were not discriminated.

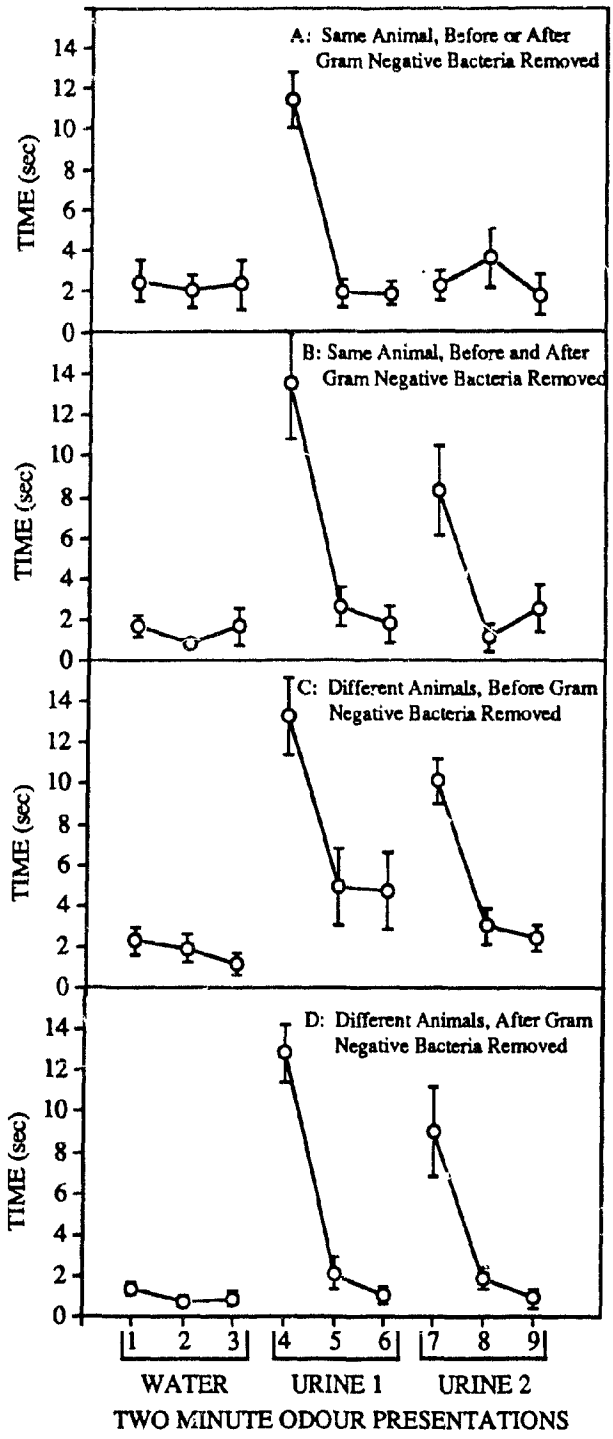


Figure 8.1. Mean (± sem) time spent sniffing urine odours from (A) the same rat before or after its gram negative bacteria were removed; (B) the same rat before and after its gram negative bacteria were removed; (C) two different rats before their gram negative bacteria were removed and (D) two different rats after their gram negative bacteria were removed.

In contrast, the subjects did discriminate between the urine odours of the same rat when the first urine sample was collected before the removal of the gram negative bacteria and the second urine sample was collected after the removal of the gram negative bacteria (Figure 8.1B). There were significant differences among the means for all eight subjects [$F(8, 56) = 16.9$, $p < 0.001$] and more time was spent sniffing the odour samples on trials 4 and 7 than on any other trials ($p < 0.01$). Hence, the urine odours of individual rats had changed after the elimination of their gram negative bacteria.

Subjects also discriminated between the urine samples of two rats collected before their gram negative bacteria were removed (Figure 8.1C). There were significant differences among the means for all seven subjects [$F(8, 48) = 14.6$, $p < 0.001$] and more time was spent sniffing the odour samples on trials 4 and 7 than on any other trials ($p < 0.01$).

Similarly, subjects discriminated between samples collected from two different rats after their gram negative bacteria were removed (Figure 8.1D). There were significant differences among the means for all eight subjects [$F(8, 56) = 20.8$, $p < 0.001$] and more time was spent sniffing the odour samples on trials 4 and 7 than on any other trials ($p < 0.01$).

If the odours of individuality had been eliminated by the removal of gram negative bacteria, two samples from two different individuals would not have been discriminable (Figure 8.1D. Thus, it would appear that although the removal of gram negative bacteria altered the individual odours of rats (Figure 8.1B), it did not eliminate their odours of individuality.

EXPERIMENT 4

THE EFFECT OF THE REMOVAL OF GRAM POSITIVE GUT BACTERIA ON THE PRODUCTION OF ODOURS OF INDIVIDUALITY IN RATS

Since removal of gram negative bacteria altered but did not eliminate individual odours, we examined the effect of removal of gram positive bacteria.

METHOD

Subjects

Thirty-two male Sprague Dawley rats were used as subjects. They were individually housed, provided with ad lib Purina Lab Chow and water and maintained on a 12:12 reversed L:D cycle, with lights off at 9:00 a.m.

Urine Donors

Urine was collected from six month old male Long Evans rats (n = 6) using the procedures described in Experiment 3.

Antibiotic Treatment

To eliminate gram positive gut bacteria, Mycifradin (Oral solution of Neomycin Sulfate 125 mg/ml) and Bacitracin (Sigma) were given in a concentration of 2.5 mg/ml in

the drinking water for two weeks as recommended by Dr. W. Hofstra, Laboratory for Medical Microbiology, University of Groningen, The Netherlands. The procedures for cage changes and food and water supplies were the same as those described in Experiment 3.

Bacteria Counts

Fecal analysis two weeks after the initiation of the drug regimen revealed that the gram positive bacteria were absent in five of the six donors (Table 8.2) and that the gram negative bacteria were still present (Table 8.3). A second urine collection from these five rats was then undertaken.

Apparatus and Procedure

The apparatus and procedure used in Experiment 3 were used in this experiment. Subjects were tested with one of four different pairs of urine samples:

A) two samples from the same rat collected on different days before its gram positive bacteria were removed or two samples from the same rat collected after its bacteria were removed (8 subjects tested);

B) urine odours of the same rat before and after its gram positive bacteria were eliminated (8 subjects tested);

C) urine odours from two different rats before their gram positive were eliminated (7 subjects tested);

D) urine odours from two different rats after their gram positive bacteria were eliminated (8 subjects tested).

Table 8.2. Gram positive bacteria count in feces of rats treated with Bacitracin and Neomycin Sulfate (2.5 mg/ml).

	Before	After
Rat TD2	400,000 col/ml	0
Rat TD3	300,000 col/ml	0
Rat TD4	350,000 col/ml	0
Rat TD5	400,000 col/ml	46,000 colonies/ml
Rat TD6	350,000 col/ml	0
Rat TD7	200,000 col/ml	0

Table 8.3. Gram negative bacteria count in feces of rats treated with Bacitracin and Neomycin Sulfate (2.5 mg/ml)

	Before	After
Rat TD2	169,000 col/ml 100 % lactose +	350,000 col/ml 100 % lactose +
Rat TD3	110,000 col/ml 100 % lactose +	1,620,000 col/ml 100 % lactose -
Rat TD4	580,000 col/ml 100 % lactose +	700,000 col/ml 40 % lactose +
Rat TD5	236,000 col/ml 100 % lactose +	19,000 col/ml 98 % lactose +
Rat TD6	190,000 col/ml 99 % lactose +	8,000 col/ml 100 % lactose +
Rat TD7	241,000 col/ml 99 % lactose +	10,000,000 col/ml 100 % lactose -

Statistical Analysis

A randomized block ANOVA was used to examine differences in the amount of time spent sniffing the odour samples over the nine trials. Newman-Keuls post hoc comparisons were used to determine which means differed from each other.

RESULTS

The results of this experiment parallel those of Experiment 3 in which the rats' gram negative gut bacteria were removed. When rats were presented with different samples from an individual rat prior to or following the removal of its gram positive bacteria. There were significant differences in the investigation times over the nine odour presentations [$F(8, 56) = 21.16$, $p < 0.001$] but only trial 4 differed significantly from any other trial ($p < 0.01$). Thus, subjects responded to the first urine presented after the water trials, but did not respond differently to the second urine odour from the same rat on trial 7 (Figure 8.2A).

In contrast, the subjects discriminated between the urine odours of the same rat when the first urine sample was collected before the removal of the gram positive bacteria and the second urine sample was collected after the removal of the gram positive bacteria (Figure 8.2B). There were significant differences among the means for all eight subjects [$F(8, 56) = 53.22$, $p < 0.001$] and more time was spent sniffing the odour samples on trials 4 and 7 than on any other trials ($p < 0.01$). Hence, the urine odours of the same rat had changed after the elimination of its gram positive bacteria.

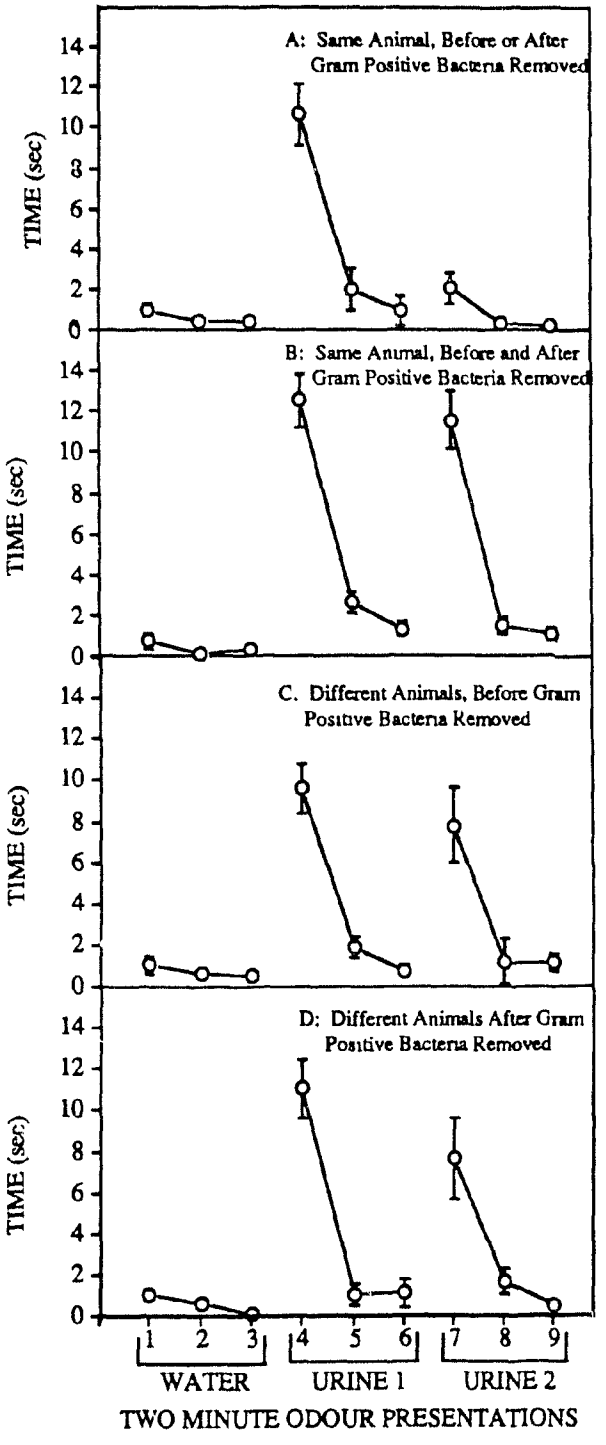


Figure 8.2. Mean (±sem) time (sec) spent sniffing urine odours from (A) the same rat before or after its gram positive bacteria were removed; (B) the same rat before and after its gram positive bacteria were removed; (C) two different rats before their gram positive bacteria were removed and (D) two different rats after their gram positive bacteria were removed.

Subjects also discriminated between the urine samples of two rats collected before their gram positive bacteria were removed (Figure 8.2C). There were significant differences among the means for all seven subjects [$F(8, 48) = 20.14$, $p < 0.001$] and more time was spent sniffing the odour samples on trials 4 and 7 than on any other trials ($p < 0.01$).

Similarly, subjects discriminated between samples collected from two different rats after their gram positive bacteria were removed (Figure 8.2D). There were significant differences among the means for all eight subjects [$F(8, 56) = 19.26$, $p < 0.001$] and more time was spent sniffing the odour samples on trials 4 and 7 than on any other trials ($p < 0.01$).

If the odours of individuality had been eliminated by the removal of gram positive bacteria, the two samples from different individuals would not be discriminable (Figure 8.2D). Thus, it would appear that although the removal of gram positive bacteria altered an individual rat's odour (Figure 8-1B), it did not eliminate its odours of individuality.

DISCUSSION

The results of these two experiments indicate that the urine odours of individual rats were altered by removal of their gram negative or gram positive gut bacteria. As the urine odours of two individual rats could still be discriminated after the removal of the bacteria, however, the removal of one type of bacteria or another was not sufficient to eliminate the odours of individuality.

To further determine the role of gut bacteria in producing unique urine odours, one could completely remove all the gut bacteria and selectively recolonize with specific bacteria. While desirable, such a task would prove difficult. My attempt to do this was not

successful as the drug treatment which resulted in the removal of the gram positive bacteria was initially undertaken to remove both gram negative and gram positive bacteria. It is possible that a different combination of antibiotics might be useful. With such success, however, comes the difficulty of collecting uncontaminated urine from these subjects.

As intestinal mucus is not degraded and water absorption is impaired because of a lack of bacteria, these animals frequently have diarrhea (Van der Waaij, 1984). Consequently, the results of testing may be difficult to interpret as the contamination of urine samples with feces may either obscure differences among samples or create artificial differences where none may exist. The recent development of ultrafine filtration products may provide a method of removing contaminants from the urine (Millipore Systems Catalog, 1993) and yield a more appropriate sample for future testing.

Based upon the results of this experiment, one would hypothesize that rats trained in the operant olfactometer to discriminate between urine samples of two individuals prior to the removal of their bacteria would not respond to probe trials of the same animals after their bacteria had been removed. If such were the case, one could consider a single probe trial in the olfactometer equivalent of the dishabituation trial in the habituation-dishabituation task. Evidence for such an equivalency would provide a way to compare the results of the two methods and potentially resolve the issue of whether or not the odours of individuality of mice are dependent upon the presence of bacteria. This hypothesis is tested in the next experiment.

CHAPTER 9

WILL RATS TESTED IN THE OLFACTOMETER BE ABLE TO DETECT CHANGES IN URINE ODOURS OF INDIVIDUALITY AFTER SELECTIVE REMOVAL OF BACTERIA?

The results of the habituation-dishabituation task (Chapter 8) provide evidence that the removal of gram negative or gram positive bacteria alters but does not remove the odours of individuality from the urine odours of rats. I wish to determine whether the same results will be obtained using the operant-olfactometer. A procedure to answer this question is to present probe trials of urine odours from donors who have had their gram negative or gram positive bacteria removed to subjects who have been previously trained to discriminate between the odours of the donors prior to the selective removal of their bacteria.

Probe trials are equivalent of the transfer of training trials used by Yamazaki et al. (1990). They refer to non-reinforced trials which are randomly presented to a subject within a session in which the subject is discriminating between known odours. If the rat perceives the probe odours as S+s they should be responded to whereas if the rat perceives the probes as S-s, they should not be responded to.

For example, in this experiment, the S+ and S- odours were from rats A (S+) and B (S-). The probe odours consisted of urine from the same individual rats, A' and B', after they were given drugs to remove their gut bacteria. Thus, if the subjects respond to A' as an S+ and B' as an S-, it would suggest that the odour of the individual is unchanged by the removal of bacteria. If, however, the subjects do not respond to the S+ probe odours, it would confirm the results of Experiments 3 and 4, which indicated that the odours of an individual were altered by the removal of gram negative and gram positive bacteria.

When evaluating the response of rats to odours as complex as those provided by urine, it may be difficult to know what a response to a probe odour means. In their Y-maze experiments, Yamazaki et al. (1990) have interpreted a response to a probe trial to mean that a mouse perceives an odour to be the same as the odours learned during discrimination training. They have interpreted a lack of response to a probe odour to mean that the probe odour is different from the training odour. Because urine is a mixture which contains many discriminable volatiles, it is my hypothesis that the responses to a probe odour may mean that the odours are somewhat similar or somewhat but not exactly the same as the training odour.

To determine if probe odours are perceived as being completely different from odours which a rat has already learned to discriminate, one could compare the rate at which rats learn to discriminate between the probe odour donors, i.e., odours from rats already discriminated but which have their bacteria removed, with their ability to discriminate between the odours of completely unknown individuals. If the pattern of learning is similar in both instances, i.e., the odours are not readily discriminated, one could conclude that altering the bacteria has completely changed the odour of the individual. If the probe odours are more easily discriminated than the odours from the unknown individuals, one might conclude that the probe odours contained some components which were familiar to the rats, i.e., were similar to the training odours. To further advance our understanding of a rat's response to a probe trial, I conducted this experiment.

EXPERIMENT 5

RESPONSE TO URINE ODOUR PROBE TRIALS FROM RATS AFTER THEIR GRAM NEGATIVE BACTERIA ARE REMOVED

The response to probe odours of individuals after their gram negative bacteria were removed as well as a comparison of discrimination learning of odours from these donors with the learning of unknown individuals are examined in the following experiment.

The rats were trained to discriminate between the urine odours from two individuals prior to the removal of their gram negative bacteria, then tested using unreinforced probe trials from the same two individuals after their gram negative bacteria had been removed.

In addition, their response to samples of the gram-negative S+ and S- probes as odours in a discrimination task were measured and compared with the acquisition of a discrimination of odours from unknown individuals.

METHOD

Subjects

Five male Long Evans rats purchased from Charles River Canada (St. Constant, Que.) at 90 days of age were tested. They were housed individually in 48 x 27 x 16 cm polycarbonate cages with stainless steel grid covers and woodchip bedding and maintained on a reversed 12:12 light:dark (L:D) cycle, with lights off at 9 a.m. The animals were provided with water and Purina Lab Chow ad lib until ten days prior to training when their water access was limited to 15 minutes per day for the duration of the experiment.

Urine Donors

The urine samples used in this experiment were collected from the same six month old male Long Evans rats described in Chapter 8.

Apparatus and Procedure

Odour stimuli were delivered to the rat by the computer controlled olfactometer described in Section 6.2. The rats were trained to perform a go/no go sequential discrimination task in a series of four steps which culminated in their learning to discriminate between the urine odours of two individual Long Evans rats. When an animal scored a mean of 85 percent or higher on each of the ten blocks of the 200 trial session, it was transferred to another training regimen. In this procedure, rats were randomly presented with four samples collected on different days from the S+ and S- odour donors within a session to accustom them to minor differences which might be present in different samples of urine from the same individuals. Such training ensures that the subjects respond to known odours in spite of minor day-to-day differences in urine concentration or the presence of food, bedding or other contaminants from day-to-day.

After the rat re-acquired the 85 % criterion, a partial reinforcement schedule (60 %) was initiated so that the introduction of non-reinforced probe trials would not disrupt performance. When performance stabilized at 85 % correct per block per session, each rat was presented with the following three types of unreinforced probe trials within two 100 trial sessions (one session per day) during which the known odours were discriminated in a go/no go discrimination task:

(A) urine samples of the S+ donor and S- donor after their gram negative bacteria had been removed;

(B) urine samples from two rats whose odours had not been previously discriminated;

(C) novel urine samples of the S+ and S- odour donors.

Following the probe trial sessions, the subjects were given five sessions (160 trials per session) in which they were required to discriminate urine odours from the previously discriminated rats after their gram negative bacteria had been removed, i.e., from the probe odour donors, as well as urine odours from pairs of previously undiscriminated rats within each session. Two different samples of each S+ and S- stimulus were presented quasi-randomly within each session.

Statistical Analysis

A repeated measures ANOVA was used to compare the percent of responses to the three different types of probe odours as well as to compare the percent of errors in the two different types of discrimination task across the five sessions. Newman-Keuls post hoc tests were used to test for differences among means. It should be noted that the responses to the S- probes were not analysed. The rats did not respond to these odours in any task.

RESULTS

The proportion of responses to each of the three probe odours is shown in Figure 9.1. There were significant differences in the percent of responses to odours collected before and after the removal of gram negative bacteria and odours from unknown donors [$F(2,8) = 9.02$, $p<.01$]. Newman-Keuls post hoc analysis revealed that there were significantly more responses to the known S+ odours than to the novel "S+" odours and to the S+ odours with the gram negative bacteria removed ($p<.05$). The percent of responses to the

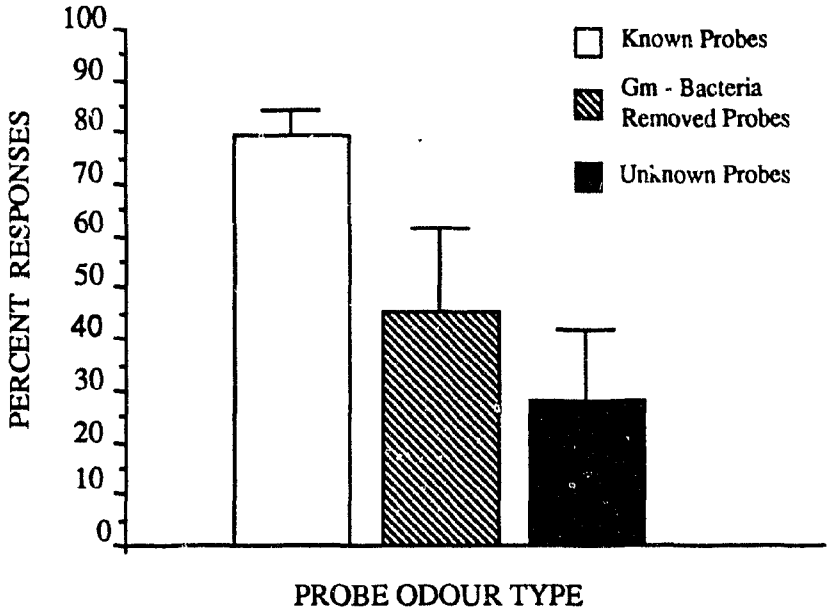


Figure 9.1. The percent of responses (±sem) to urine odour probes from known individuals, from these same individuals after their gram negative bacteria had been removed and from unknown outbred rats. Each probe odour was presented for 4-5 trials.

bacteria depleted S+ and the unknown S+ did not differ significantly. These results confirm that removal of gram negative bacteria alters the urine odours of rats.

Analyses of the number of errors to criterion indicates that subjects made significantly fewer errors in learning to discriminate between the odours of the donors after the elimination of their gram negative bacteria than between the odours of two unknown rats; however, this difference in performance was eliminated, after four training sessions, (Figure 9.2). A 2 x 5 ANOVA revealed a main effect of odour type [$F(1,4) = 16.06$, $p<.05$] a main effect of session [$F(4,16) = 11.79$, $p<.001$] and a significant interaction [$F(4, 16) = 7.02$, $p<.01$]. Newman-Keuls analysis indicated that in the discrimination of the gram negative probes, there was no further decrease in the number of errors after the second session but for the unknown probes, the number of errors decreased significantly until the third session.

Thus, after removal of the gram negative bacteria, a rat's odour can still be discriminated, as indicated by the ease with which the rats learned the discrimination of odour donors compared with the ability to discriminate between the odours from unknown individuals. The learning curve of one subject is shown in Figure 9.3 to illustrate this result. Despite the superior performance of the subjects relearning to discriminate between rats with their gram negative bacteria depleted, the ability of the rats to quickly learn and maintain a completely new discrimination should also be noted.

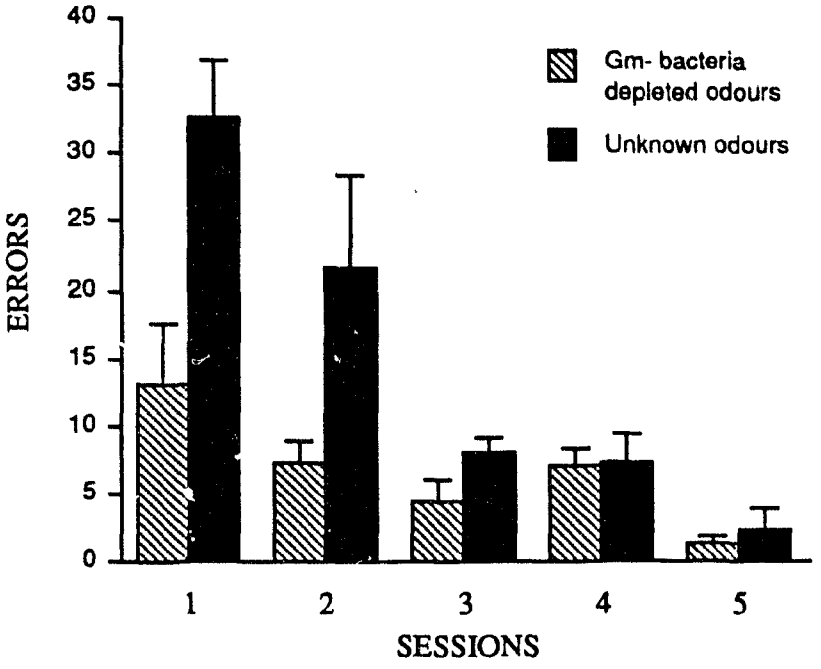


Figure 9.2. The mean (± sem) number of errrors made by five rats in learning to discriminate between the urine odours of previously discriminated outbred rats after their gram negative bacteria had been removed (▨) and between the urine odours of previously undiscriminated outbred rats (■).

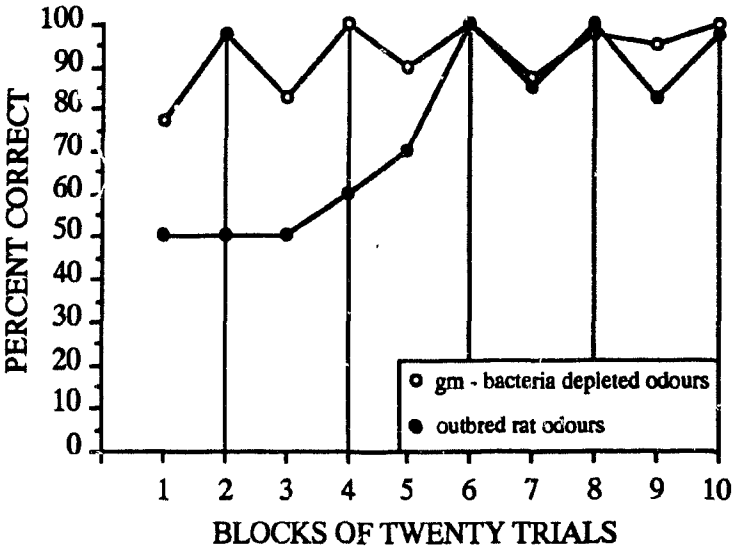


Figure 9.3. The learning curve of one rat discriminating between the urine odours of previously discriminated rats after their gram negative bacteria had been removed (O) and discriminating between the urine odours of previously undiscriminated rats (●). Two samples of each S+ and S- were presented. For clarity, the mean responses to each odour type are presented.

EXPERIMENT 6

RESPONSE TO URINE ODOUR PROBE TRIALS FROM RATS AFTER THEIR GRAM POSITIVE BACTERIA ARE REMOVED

The response to probe odours of individuals after their gram positive bacteria were removed as well as a comparison of discrimination learning of odours from these donors with the learning of unknown individuals are examined in the following experiment.

METHOD

Subjects and Urine Donors

The five Long Evans rats trained in the operant-olfactometer for Experiment 9-A also learned to discriminate between the urine from different pairs of outbred Long Evans rats prior to the removal of their gram positive bacteria. The procedure for removal of the gram positive bacteria is described in the methods section of Chapter 8.

Procedure

The training procedure utilized in Experiment 5 was also followed in this experiment, i.e.re subjects were presented with non-reinforced probe odours following successful performance on a session in which four samples collected on different days from both the S+ and S- odour donors were presented randomly on a 60 % partial reinforcement schedule. The following types of probe trials were presented within two 100 trial sessions

(one session per day) during which the known odours were discriminated in a go/no go discrimination task:

(A) urine samples of the S+ donor and S- donor after their gram positive bacteria had been removed;

(B) urine samples from two rats whose odours had never been previously discriminated;

(C) novel urine samples from the S+ and S- odour donors.

Following the probe trial sessions, the subjects were given five sessions (160 trials per session) in which they were required to discriminate urine odours from the previously discriminated rats after their gram positive bacteria had been removed, i.e., from the probe odour donors, as well as urine odours from pairs of previously undiscriminated rats. As in Experiment 5, two different samples of each stimulus were presented within each session. As the S+ and S- odours were inadvertently reversed for one of the rats on the first of these sessions, its results were not included in the analysis.

Statistical Analysis

A repeated measures ANOVA was used to compare the percent of responses to the three different types of probe odours as well as to compare the percent of errors in the two different types of discrimination task across five sessions. Newman-Keuls post hoc comparisons were used to test for differences among means. The responses to the S- probes were not analysed.

RESULTS

The number of responses to each of the three odour types of probe odours as shown in Figure 9.4. There were significant differences among the percent of responses to known probe odours, odours from the donors after removal of the gram positive bacteria and odours from unknown donors [$F(2,8) = 17.47$, $p<.001$]. Newman-Keuls post hoc analysis revealed that there were significantly more responses to the known S+ odours than to the unknown "S+" odours and to the S+ odours with the gram positive bacteria removed ($p < .05$). The percent responses to the bacteria depleted S+ and the unknown S+ did not differ significantly. These results confirm that removal of gram positive bacteria alters the urine odours of rats.

Analysis of the errors to criterion indicates that the rats made significantly fewer errors in learning to discriminate between the odours of the donors after the elimination of their gram positive bacteria than between the odours of two unknown rats (Figure 9.5). A 2 x 5 ANOVA revealed a main effect of odour type [$F(1,3) = 12.37$, $p<.05$] but no effect of session [$F(4,12) = 1.97$] and no significant interaction [$F(4, 12) = 2.99$]. Thus, after removal of the gram positive bacteria, discriminable components of a rat's odour still remain as indicated by the ease with which the rats learned to discriminate between these odour donors compared with their ability to discriminate between the odours from unknown individuals.

Only two of the four rats were able to meet the criterion of 85 % per block for both odours within a session when discriminating between the odours from unknown subjects. The other two rats were able to meet this learning criterion within eight sessions. As shown in Figure 9.6, the learning curves for a "good" subject and a "bad" subject illustrate this result.

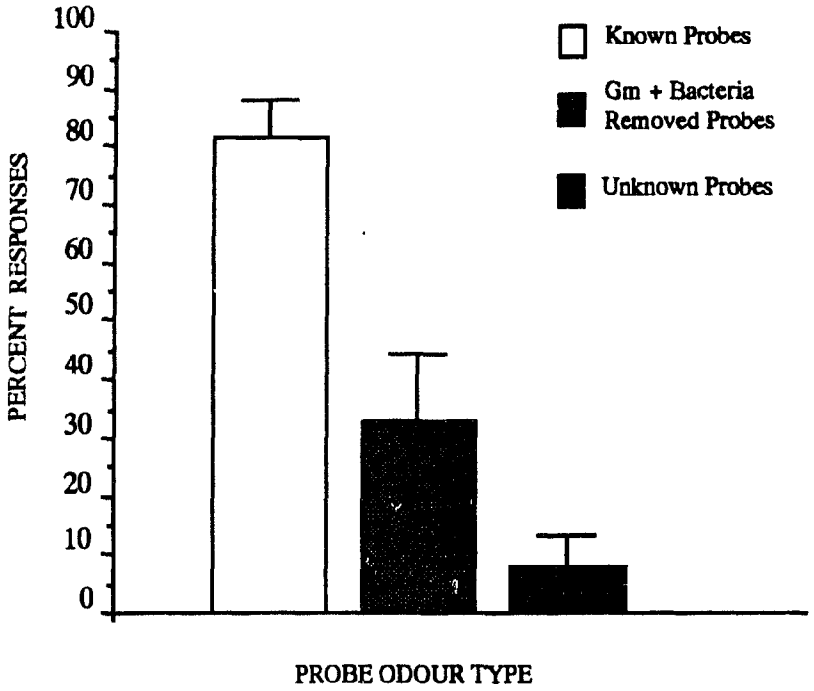


Figure 9.4. The percent of responses (±sem) to urine odour probes from known individuals, from these same individuals after their gram positive bacteria had been removed and from unknown outbred rats.

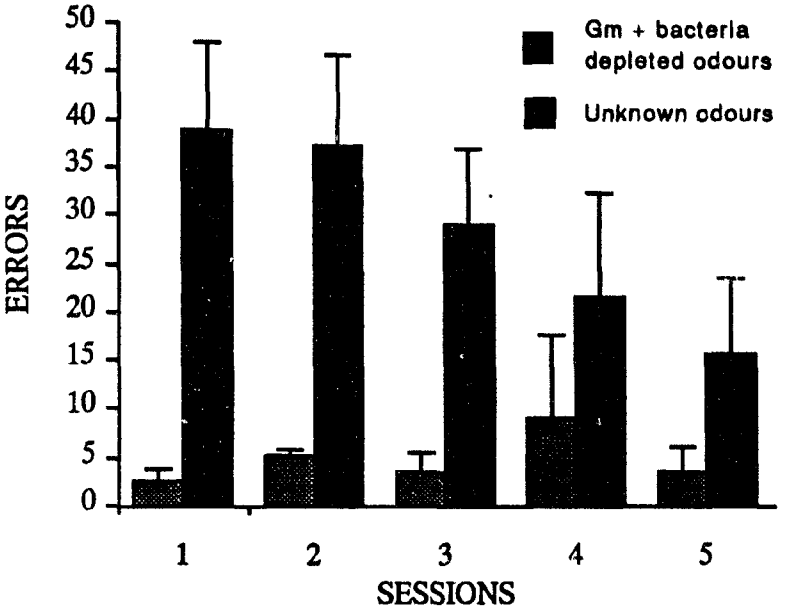


Figure 9.5. The mean (±sem) number of errrors made by five rats in learning to discriminate between the urine odours of previously discriminated outbred rats after their gram positive bacteria had been removed (■) and between the urine odours of previously undiscriminated outbred rats (■).

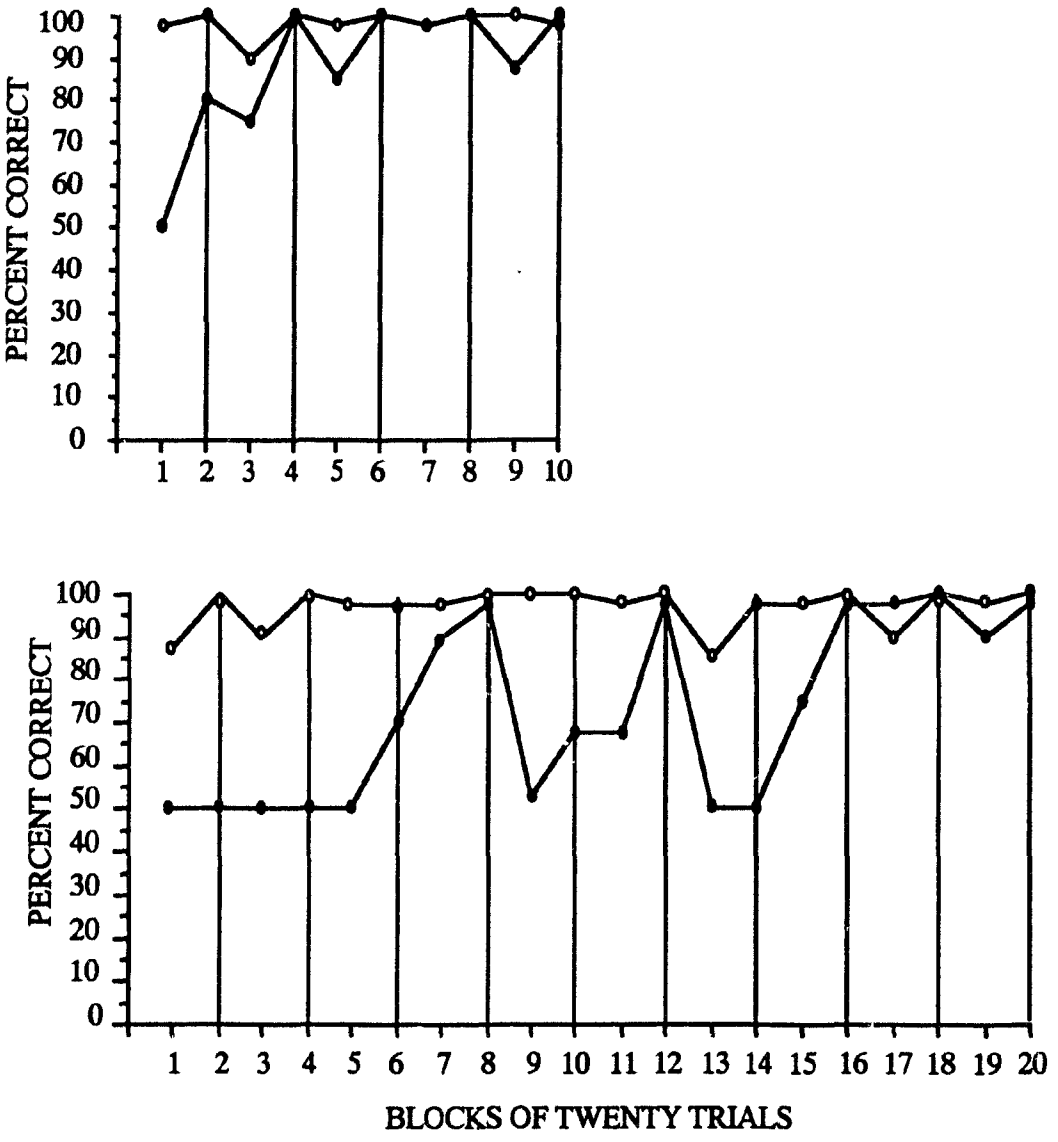


Figure 9.6. The learning curve of two rats discriminating between the urine odours of previously discriminated rats after their gram negative bacteria had been removed (O) and discriminating between the urine odours of previously undiscriminated rats (●). (A).One rat met criterion on both tasks within five sessions; (B) the other rat completed both tasks within ten sessions. Two samples of each S+ and S- were presented. For clarity, the mean responses to each odour type are presented.

DISCUSSION

The results of these two experiments confirm that removal of the gram negative and gram positive bacteria from the gut of rats alters their odours of individuality. Rats did not respond to urine odour probes after elimination of specific groups of bacteria any differently than they did to probe odours from unknown individuals.

Nonetheless, a comparison of the ability of subjects to learn to discriminate between the two different types of urine odour probes indicated that it was easier to learn to discriminate between the odours of the previously known individuals after their bacteria had been altered than it was to discriminate between the odours of unknown individuals. This "savings" effect demonstrates that the even though the odours of individuals were altered by the elimination of gram negative or gram positive bacteria, they still retained some components which were useful in identifying them as familiar and made them easier to discriminate than the unknown odours. These results suggest that the lack of response of rats to probe odours should not always be taken as conclusive evidence that the odours are perceived as different from previously learned odours. One might hypothesize that the converse is also true, i.e., responses to probe odours may mean that the odours are similar in some but not all dimensions.

This finding may be particularly relevant to the discrimination of mixtures such as urine. Because of the complexity of such a mixture, it appears that multiple cues are available. Consequently, in some instances, a discrimination may be based upon genetic cues and in others it may be based upon cues provided by dietary factors. While the rat may be using specific cues, e.g., genetic cues, to make a discrimination, it is possible that background differences, e.g., diet cues, are automatically processed. Such cues could be primed for recognition in an instance where the previously used cues are no longer

available and other cues must be used. This could result in faster learning in a situation such as the one provided in this experiment.

While the results of the Experiments 5 and 6 were similar, there was one particular discrepancy. In the experiment in which the gram negative bacteria were removed, all of the subjects reached a high level of performance in discriminating between both the odours from gram negative depleted individuals and the odours from unknown conspecifics within five sessions. In contrast, when learning to discriminate between the odour pairs from gram positive depleted individuals and those from unknown conspecifics, only half of the subjects were at the same level of performance on both discrimination tasks after five sessions. There is no obvious explanation for this result. The stimuli in question were discriminated by the other subjects so the possiblity that the odours were difficult to discriminate can be discounted. Moreover, the rats were able to discriminate between the other test stimuli after being trained for three more sessions so it can not be a result of their inability to perform the task.

In both probe trial experiments, the mean responses to the bacteria depleted odours and the unknown odours did not differ significantly (Figures 9.1 & 9.4). Nonetheless, there was a fair amount of variance in the responses to the bacteria depleted odours. In both experiments, this variance resulted from one subject (a different rat in each case) responding to the bacteria depleted odours as if they were known odours. The rat may have responded in this fashion because it perceived the odours to be similar to the known odours or because of its particular response strategy. For example, one of these rats responded to the odour from a known conspecific, an unknown individual and the known donor after removal of its gram negative bacteria as if they were an S+. As there is no punishment for responding incorrectly to an odour presentation, this would be the most appropriate strategy to gain the maximum amount of water reinforcement in an ambiguous

situation. Nonetheless, most rats did not adopt this strategy when presented with probe trials. If they were presented with a novel odour, they did not respond.

The comparison of the rate of learning in the discrimination tasks directly following the probe trial sessions has demonstrated unequivocally that the odours of the bacteria depleted rats are easier to learn than the odours from unknown rats. Indirectly, these results indicate that the odours are only somewhat different from the odours of the donor rats prior to the removal of their bacteria. If the subjects had perceived the bacteria-depleted odours as being totally different from the odours of these same rats before the depletion of the bacteria, one would have expected their response to these odours to be no different than their response to the novel odours, i.e., they would initially be difficult to learn.

The addition of these discrimination tasks following the presentation of probe trials has significantly contributed to our understanding of a rat's responses to probe trials. I have confirmed that a lack of response to a probe trial or transfer of training trial does not mean that the odours are necessarily completely different from the odours presented in the training sessions. Consequently, it would seem appropriate to include a similar procedure in any further analysis of probe trial data.

Experiments 5 and 6 have also shown that bacteria are important for the production of individual odours in rats. Since bacteria act on a dietary substrate, the next experiments examined the effects of changes in diet on individual odours.

CHAPTER 10

EXPERIMENT 7

ARE RATS ABLE TO DISCRIMINATE BETWEEN THE URINE ODOURS OF GENETICALLY IDENTICAL MICE MAINTAINED ON DIFFERENT DIETS?

Dietary changes also alter the odours of individuals (Beauchamp, 1976; Skeen & Thiessen, 1977; Galef, 1981; Brown & Wisker, 1989). To account for the finding that, in rats, bacteria interact with the genetic differences at the MHC to produce individually distinct urinary odours, Singh et al. (1990) hypothesized that the gastrointestinal bacteria act on dietary products to produce a pool of volatile metabolites which are carried to the urine by the class I MHC antigens.

The urine of germfree rats is qualitatively and quantitatively different from that of conventionally reared rats (Holland et al., 1983) and since some urinary components are formed by the action of gastrointestinal bacteria on dietary products (e.g. Peppercorn & Goldman, 1972), I hypothesized that dietary changes might alter individual urinary odours independently of genetic differences. To test this, I examined the effects of diet on individual odours in genetically identical mice in the habituation-dishabituation task.

METHOD

Subjects

The subjects were 24 male Long Evans hooded rats purchased from Charles River (Quebec) when 70-90 days of age. They were housed individually in 25 x 19.5 x 18.5 cm Wahman hanging cages in a colony room on a 12:12 reversed light:dark cycle, with lights off from 8 am to 8 pm. Purina lab chow and water were provided *ad lib* throughout the experiment. Each subject was tested when about 120 days of age.

Urine Donors

The urine donors were C57BL/6J mice born in our laboratory to mated brother/sister pairs purchased from the Jackson Laboratory (Bar Harbour, Maine). The urine donors thus had an H-2^b MHC (H-2) type. The urine donors were weaned at 21 days of age and housed in all male littermate groups until 70 days of age when they were housed individually in 29 x 18 x 12.5 cm plastic cages with wood chip bedding. All mice were housed in a colony room on a 12:12 reversed light:dark cycle, with lights off from 11:30 am to 11:30 pm.

Parents were placed on one of two diets before breeding and maintained on one of these diets, either Purina Lab Chow (Diet P) or Hagan Hamster food (Diet H). The constituents of the diets in gm/Kg are as follows: Purina: 253 -crude protein, 48 - fat, 581- carbohydrate, 10.2 calcium, and 8.4 - phosphorous; Hagen: 147 - crude protein, 159 - fat, 652 - carbohydrate, 2.8 - calcium, and 4.2 - phosphorous. The Purina diet has an

energy value of 4072 calories per gram while the Hagen diet has an energy value of 4802 calories per gram.

Urine Collection

Urine samples were collected daily from six different individual male mice on each diet during the light phase of the L:D cycle. Samples were obtained by holding the mouse over a collecting vial and gently stroking the abdomen above the bladder. This method guaranteed fresh urine samples which were not contaminated by feces. Samples were identified by donor and collection date and stored in 1.5 ml Canlab collection vials and frozen at -20^{o} C until required.

Apparatus and Procedure

The apparatus and procedure used in the habituation-dishabituation paradigm is fully described in Chapter 6. Each subject was tested once in a series of nine 2-min odour presentations, three with water, three with urine sample A and three with urine sample B. Each group of eight subjects received one of three different urine odour pairs. These were urine from: (1) two male mice on different diets; (2) two male mice on the same diet or (3) two samples from the same male mouse. In tests one and two, half of the subjects received urine samples in the order A-B and half in the reverse order, B-A. In the third test, half of the subjects received urine from mice on the Purina diet and half received urine from mice on the Hagen diet. All tests were conducted under dim white light during the dark phase of the light-dark cycle.

Statistical Analysis

A randomized blocks ANOVA was used to examine differences in time spent sniffing the odour samples over the nine trials and Newman-Keuls post-hoc tests were used to determine which means differed from each other.

RESULTS AND DISCUSSION

Rats could discriminate between the urine odours of C57BL/6J male mice on two different diets (Figure 10A). There were significant differences among the nine means for all eight subjects ($F(8,56) = 5.84$, $p<.001$) and more time was spent sniffing the odour samples on trials 4 and 7 than on any other trials ($p<.05$). Hence, the two urinary odours were discriminable.

Rats could not discriminate between the urine odours of individual mice on the same diet (Figure 10B). There were significant differences among the nine means for all eight subjects ($F(8, 56) = 10.68$, $p<.001$), but only trial 4 significantly differed from any other trial ($p<0.05$). Thus, subjects dishabituated when presented with the first urine after the water presentations but did not respond differently to the second urine sample from a different individual maintained on the same diet. Likewise, rats did not increase their sniffing time when presented with the second of two urine samples from the same individual maintained on either Diet P or Diet H (Figure 10C). There were significant differences among the nine means for all eight subjects ($F(8,56) = 16.02$, $p<.001$). Only on trial 4 did the investigative time significantly differ from any other trial ($p<0.05$).

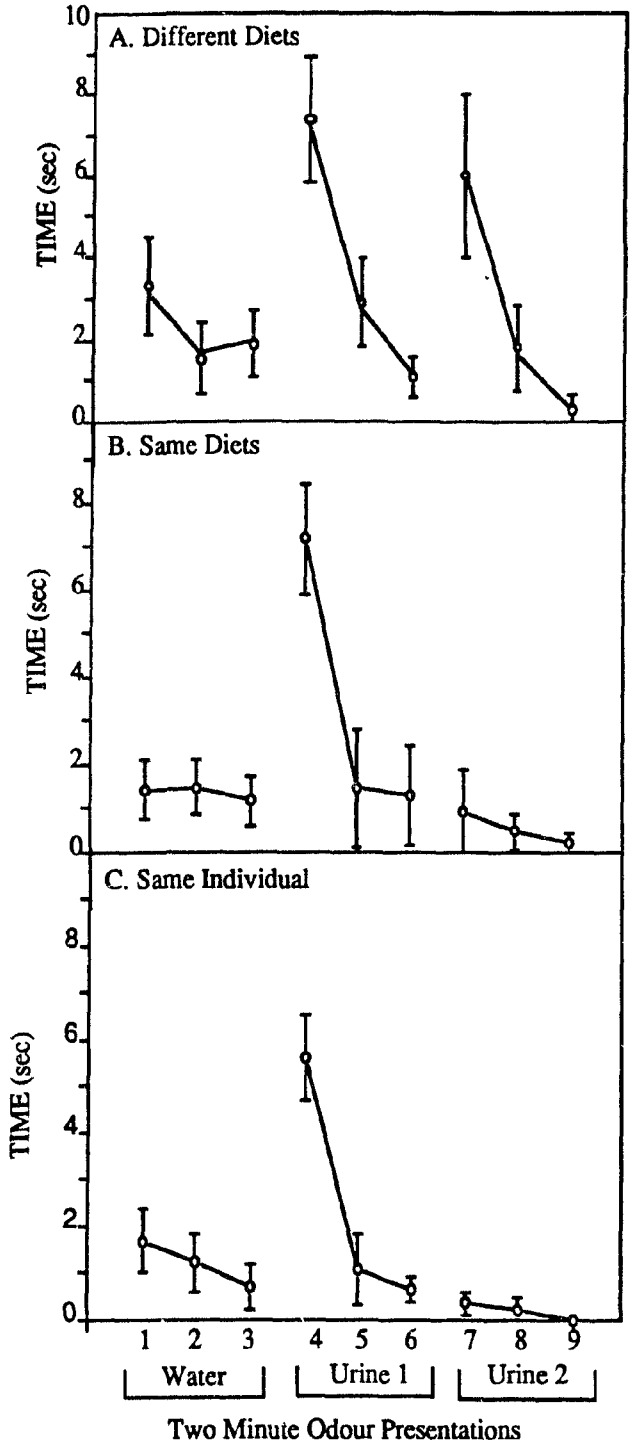


Figure 10. Mean (± sem) time (sec) spent rearing and sniffing urine odours from: (A) two genetically identical mice on different diets; (B) two genetically identical mice on the same diet and (C) two urine samples from the same individual mouse.

The results indicate that male Long Evans hooded rats are able to discriminate between the urine odours of genetically identical mice maintained on different diets and that this discrimination can be made regardless of day-to-day variations in the odours of individuals which may occur due to physiological or environmental changes. Consequently, diet should be taken into account when considering the factors involved in the production of unique individual odours. The two diets used differ in their five main nutrients and in their energy value. It is possible that these differences produce variations in the metabolites excreted in the urine, thus contributing to individual differences in urinary odours and conveying information on individuality.

It is well known that specific nutrients can create a distinctive urine odour. For example, in humans, the consumption of asparagus produces the odour of rotten cabbage in the urine (Mitchell, 1989) and disorders of amino acid metabolism such as phenylketonuria and maple syrup urine disease result in characteristic urine odours (Liddell, 1976). The elimination of these urine odours after dietary changes to reduce the intake of the foods involved emphasizes the role of diet in creating distinctive urinary odours.

It seems unlikely that a dietary factor alone could provide a consistent mechanism for individual recognition. Indeed, the results of previous studies suggest that individual urinary odours are produced primarily by genetically controlled variations (Beauchamp et al, 1990). An environmental factor such as diet would vary according to season and locale in which an individual resides. Genetic components would not be subject to such variability. However, odour production may be strongly affected by diet (Beauchamp, 1976, Galef, 1981) and variations in diet may convey information of value in the recognition of individuals. Differences in diet may also be useful in the identification of

kin because individuals would be more familiar with an odour which reflects a diet similar to their own.

In order for olfactory cues to be used for individual or kin recognition, by phenotype matching, the odours should provide a consistent representation of the genetic similarities between individuals (Lacy & Sherman, 1983; Waldman, 1987; Waldman, Frumoff, & Sherman, 1988). Odour differences based on genetic differences between individuals provide a direct mechanism for individual or kin recognition while odour differences based on dietary differences would provide an indirect mechanism for individual or kin recognition (Waldman, 1987) and both of these mechanisms may act together.

The urine odour of individuality appears to be determined by a complex interaction of genetic, microbiological and dietary factors. Although the evidence is mixed that bacteria are essential for the production of the unique urinary odours in mice, as occurs in rats (Yamazaki et al., 1990; Chapter 7), the fact that diet alters urine odours as well as feces odours in mice (Brown & Wisker, 1989) indicates that factors other than genetic differences must be active in producing discriminable differences in the urinary odours of individual mice. This suggests that an unchanging genetic component in conjunction with certain environmental factors may be operating to produce urinary odours of individuality. It is not known how these factors interact to produce a unique odour. This issue will be addressed in Chapter 11.

CHAPTER 11

DO DIETARY AND GENETIC CUES INTERACT IN THE PRODUCTION OF URINE ODOURS OF INDIVIDUALITY OF MHC CONGENIC MICE?

Changes in diet alter the maternal odours of rats (Leon, 1974, 1975), gerbils (Skeen & Theissen, 1977) and mice (Doane & Porter, 1978; Brown & Wisker, 1989) and the urine odours of guinea pigs (Beauchamp, 1976). The urine odours of MHC congenic strains of mice maintained on the same diet can be discriminated (Yamazaki et al., 1983) as can the odours of genetically identical mice maintained on different diets (Chapter 10). Thus, we know that both the MHC and the diet can independently influence the urine odours of mice but we do not know how these factors interact. To investigate the interaction of genetic and dietary factors on the production of individual odours in mice, I tested Long Evans hooded rats for their ability to discriminate between the urine odours of two strains of MHC congenic mice maintained on two different diets.

EXPERIMENT 8

DO DIETARY AND GENETIC CUES INTERACT IN THE PRODUCTION OF URINE ODOURS IN MHC CONGENIC MICE WHICH DIFFER AT ONE LOCUS?

METHOD

Subjects

Sixteen male Long Evans hooded rats purchased from Charles River Canada Inc. (St. Constant, Quebec) at 70 - 90 days of age were used as subjects. They were housed individually in 48 x 27 x 16 cm polycarbonate cages with stainless steel grid covers and woodchip bedding and maintained on a reversed 12:12 light:dark (L:D) cycle, with lights off at 8 a.m. The animals were provided with Purina Lab Chow and water ad lib. Ten days prior to training, their water intake was restricted to 10-15 ml per day for the duration of the experiment.

Urine Donors

Urine was collected from twenty male C57BL/6-H-2K^{b}/J (BL6) and C57BL/6-H-2K^{bml}/ByJ (BMl) mice bred from brother/sister pairs purchased from the Jackson Laboratory (Bar Harbour, Maine). These two strains differ at only one locus of the MHC (the H-2K locus) and previous studies have shown that their urine odours can be

discriminated by other mice (Yamazaki et al., 1990) and by rats (Beauchamp et al., 1990). All urine donor mice were housed individually in 29 x 18.5 x 12.5 cm plastic cages with woodchip bedding in a mouse colony room on a reversed 12:12 L:D cycle, with lights off at 11:30 a.m.

Half of the mouse urine donors of each strain were maintained on Purina Lab Chow (Diet P) and the other half on Hagan Hamster food (Diet H). The constituents and energy value of these diets are described in Chapter 10. The dams of these mice were placed on the two different diets at the time of mating so the pups, which were the odour donors, were on the different diets since birth. Urine was collected when the mice were 3-9 months of age.

Urine Collection

Urine samples were collected daily at the end of the light phase by gently stroking the abdomen (slightly above the bladder) of each donor. Samples from the same individual were pooled over several days to provide sufficient urine for a 0.5 ml sample and were stored in 1.5 ml Canlab storage vials, labelled with the donors identification number, dated, and frozen at -20^{o} C until required.

Apparatus and Procedure

Odour discrimination was evaluated in the operant olfactometer described in Chapter 6. Following pretraining procedures which culminated in an odour-no odour discrimination, the subjects were trained to discriminate between urine odours from four

pairs of outbred rats in succession to ensure that they were proficient in a two odour discrimination task prior to learning to discriminate between the mouse urine odours.

Discrimination of mouse odours. Upon the completion of the rat odour discrimination training, four subjects were tested for their ability to discriminate between each of the following four pairs of mouse odours: Task 1: Urine from individual mice of the BL6 versus BMl strains maintained on the same diet. Task 2: Urine from individual mice of the BL6 versus BMl strains maintained on different diets. Task 3: Urine from individual mice which were genetically identical and maintained on the same diet. Task 4: Urine from individual mice which were genetically identical and maintained on different diets.

For each task, the designation of odour stimuli as S+ or S- was counterbalanced for strain and diet type. For example, in Task 1, two subjects received presentations of urine samples from BM1 and BL6 maintained on Diet H, with one subject receiving the BM1(H) urine as the S+ and the other receiving the BL6(H) urine as the S+. Two subjects received presentations of urine samples from BM1 and BL6 mice maintained on Diet P, with one subject receiving the BM1(P) urine as the S+ and the other receiving the BL6 (P) urine as the S+.

In each task, subjects were tested until they met the learning criterion of 85% correct on the first block of trials and a mean of 85% on the remaining nine blocks for three consecutive sessions for a maximum of ten sessions. The requirement for meeting the criterion over three consecutive sessions was chosen for two reasons: first, to determine if the subjects were using a constant cue which could be remembered from session to session and, second, to ensure that learning was not due to differences in urine concentration or

intensity or any other subtle day-to-day variation that might have existed between odour stimuli within a single session.

Statistical Analysis

The sessions to criterion, errors to criterion and first block errors were first analyzed using a one-way analysis of variance (ANOVA). Next, I used analysis of covariance (ANCOVA) to analyze the data. The extensive pretraining experience in discriminating outbred rat odours was intended to eliminate differences in individual learning ability; however, in case the subjects' previous experience influenced their performance, I undertook the following three analyses: first, to analyze the number of sessions to criterion, I used the number of sessions to reach criterion on the final pair of outbred rat odour discriminations as a covariate; second, to analyze the number of errors to criterion, the covariate used was the mean number of errors per session on the last pair of outbred rat odours; third, to analyze the number of first block errors to criterion, I used the mean number of first block errors per session on the last pair of outbred rat odours as the covariate. None of the results of the ANCOVAs differed from the results of the ANOVAs, so I have reported only the ANOVA results and the results of planned comparisons.

RESULTS

Sessions to criterion. Only one of the four subjects learning Task 1 met the criterion for discriminating between the urine odours of MHC congenic mice on the same diet. However, one other subject met the criterion on two non-sequential sessions. All four subjects learning Task 2 were able to discriminate between the urines of MHC congenic

mice maintained on different diets. No subjects learning Task 3 were able to meet the criterion for discrimination between the urine odours of genetically identical mice maintained on the same diet. All four subjects learning Task 4 could discriminate between the urines of genetically identical mice maintained on different diets. Figure 11.1 shows the learning curves for a representative subject from each of the four tasks.

The number of daily sessions required by each subject to reach the learning criterion are shown in Table 11.1. Subjects not meeting criterion were tested for a maximum of ten sessions and given a score of 10 as the number of sessions to criterion. An ANOVA revealed significant differences in the number of sessions to criterion over the four tasks: [$F(3,12) = 4.34$, $p < .05$]. Three planned contrasts tested specific hypotheses about the differences among the four tasks. The number of trials to learn Task 2 (different strain, different diet) did not differ from the number of trials to learn Task 4 (same strain, different diet) [$F(1,12) = .07$)], indicating that changing the strain of mice as well as the diet did not make it easier to learn the discrimination.

Task 1 (different strain, same diet) took significantly more trials to learn [$F(1,12) = 4.94$, $p = .05$] than Task 2 (different strain, different diet) and Task 4 (same strain, different diet). This indicates that learning to discriminate mice on different diets is easier than learning to discriminate mice on the same diet, whether the mice are genetically different or the same.

Task 3 (same strain, same diet) took significantly longer to learn [$F(1,12) = 8.01$, $p < .05$] than the combined conditions of Task 1 (different strain, same diet), Task 2 (different strain, different diet) and Task 4 (same strain, different diet). Thus, it was more

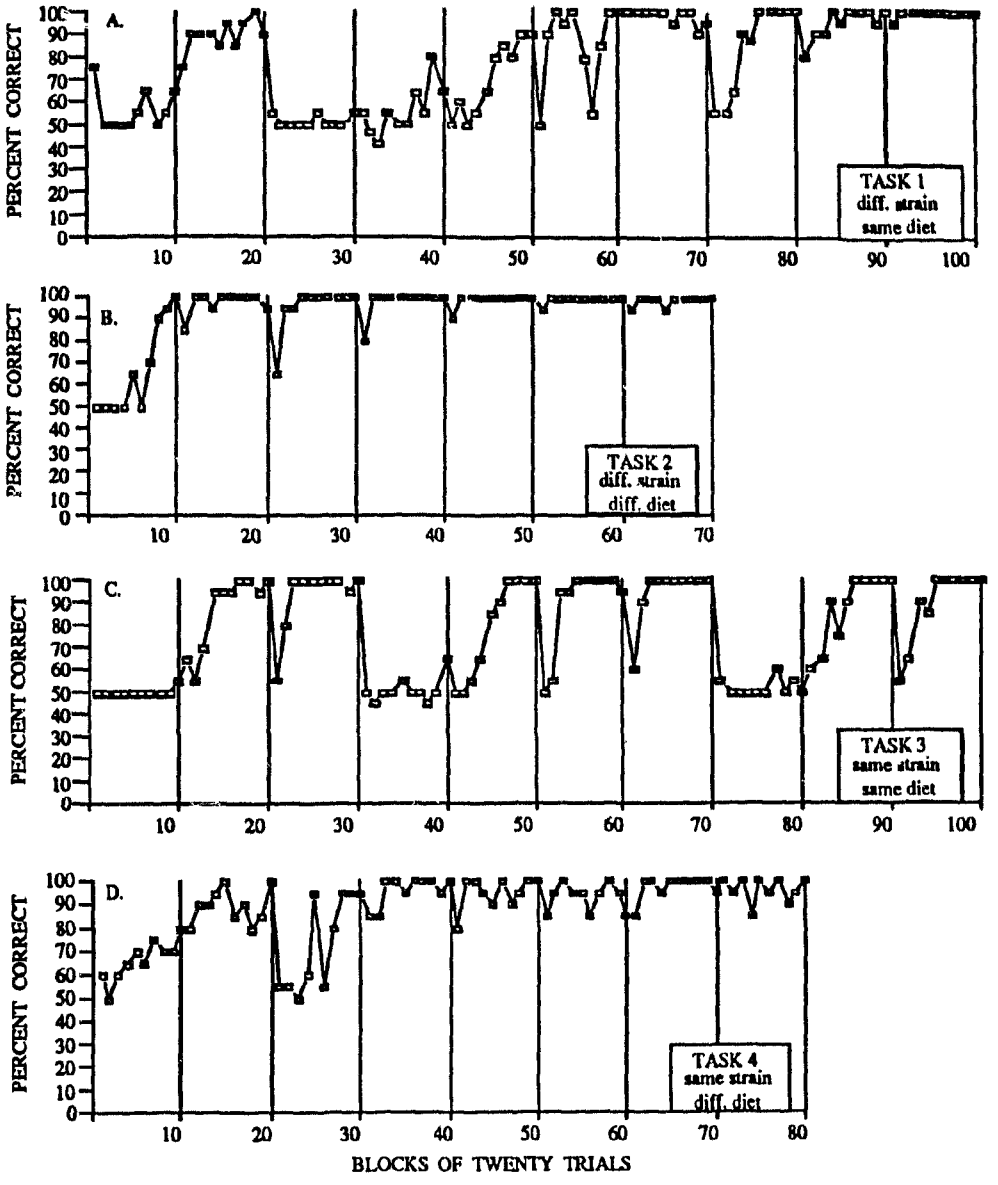


Figure 11.1. The percent correct responses for a representative subject from each of the four groups tested for their ability to discriminate between the urine odours of the following: A: mice of two different MHC congenic strains on the same diet (Task 1); B: mice of two different MHC congenic strains on different diets (Task 2); C: two mice of the same strain on the same on the same diet (Task 3) and D: two different mice of the same strain on different diets (Task 4). Ten blocks of 20 trials (200 trials) were presented on each daily session. Each data point represents one block of 10 S+ and 10 S- trials.

Table 11.1: The number of daily sessions required for each of the four subjects in each task to meet the learning criterion in the discrimination of urine odours from MHC congenic mice. A maximum of ten daily sessions of 200 trials was given to each rat.

	Subject				
Task	1	2	3	4	Mean
1. Different Strain Same Diet	6	10	10	10	9
2. Different Strain Different Diet	8	8	6	6	7
3. Same Strain Same Diet	10	10	10	10	10
4. Same Strain Different Diet	8	6	9	6	7.25

difficult to learn a discrimination when animals were genetically identical and on the same diet, than when either strain or diet differed.

Errors to criterion. Although three of the subjects learning Task 1 and all of the subjects learning Task 3 failed to meet the criterion, this did not mean that they could never discriminate between any of the odour pairs within a daily test session. The data indicate that they did not remember the discrimination from session to session. For example, the rat whose performance on Task 1 is illustrated in Figure 11.1A scored 85 percent or greater on each block of trials in both sessions seven and ten but did not score 85 percent or above on three sessions in a row.

An ANOVA of the mean number of errors to criterion (or for all ten sessions for the subjects not reaching criterion) revealed significant differences among groups [$F(3,12) = 8.2$, $p < .01$] (See Figure 11.2). Planned contrasts revealed the following: (1) the number of errors in learning to discriminate between Task 2 (different strain, different diet) and Task 4 (same strain, different diet) was not significantly different [$F(1,12) = .09$) indicating that a difference in strain did not make it easier to learn to discriminate between individuals whose diet also differed; (2) the errors to criterion for Task 1 (different strain, same diet) were significantly greater [$F(1,12) = 16.5$, $p < .01$] than the errors to criterion for the combined conditions of Task 2 (different strain, different diet) and Task 4 (same strain, different diet), indicating that it was easier to learn to discriminate mice on different diets rather than mice on the same diet, regardless of whether the mice differed genetically, (3) the errors to criterion for subjects in Task 3 (same strain, same diet) were significantly greater [$F(1,12) = 7.9$, $p < .05$] than the errors to criterion for the combined conditions of Task 1 (different strain, same diet), Task 2 (different strain, different diet) and Task 4 (same strain, different diet) reflecting that it was most difficult to learn to discriminate

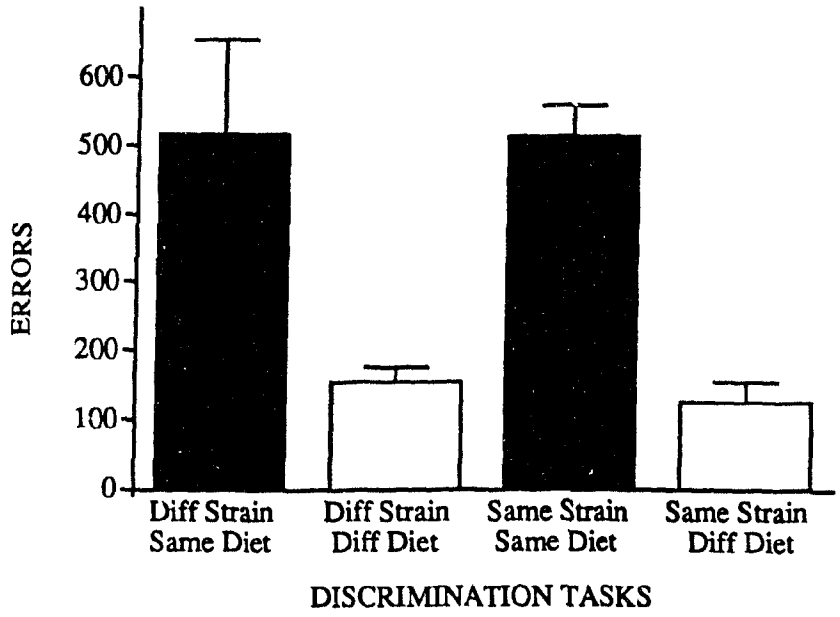


Figure 11.2. The mean (± sem) number of errors to a criterion on 85% correct for three consecutive sessions (or for a maximum of ten sessions) on each of the four urine odour discrimination tasks.

between the odours of genetically identical mice on the same diet.

First block errors. Examination of Figure 11.1 shows that rats have a within-session learning curve as well as a between-sessions learning curve, i.e., within each daily session of 10 blocks of 20 trials, the percent correct is lower on the first block and then increases within a session. Between sessions, the percent correct increases gradually over days. The number of errors on the first block of 20 trials reflects the subject's memory for the task from one session to the next.

Figure 11.3 shows the mean number of first block errors to criterion (or for all ten sessions for the subjects not reaching criterion) for each task. The ANOVA revealed significant differences among tasks [$F(3,12) = 14.39$, $p < .001$]. Three planned contrasts were undertaken. The first contrast, comparing the number of first block errors on Task 2 (different strain, different diet) and Task 4 (same strain, different diet) was not significant [$F(1,12) = .18$)]. Thus, the addition of a strain difference did not make it any easier for the rats to remember the difference between the odours of mice across sessions.

The second contrast demonstrated that the first block errors to criterion for Task 1 (different strain, same diet) were significantly greater [$F(1,12) = 15.3$, $p < .01$] than the first block errors to criterion for the combined conditions of Task 2 (different strain, different diet) and Task 4 (same strain, different diet), an indication that it is easier for rats to remember the difference between the odours of mice based upon dietary differences regardless of their genetic background.

The third contrast showed that the first block errors to criterion for subjects in Task 3 (same strain, same diet) were significantly greater [$F(1,12) = 27.96$, $p < .01$] than the first block errors to criterion for the combined conditions of Task 1 (different strain, same diet), Task 2 (different strain, different diet) and Task 4 (same strain, different diet). Thus, rats

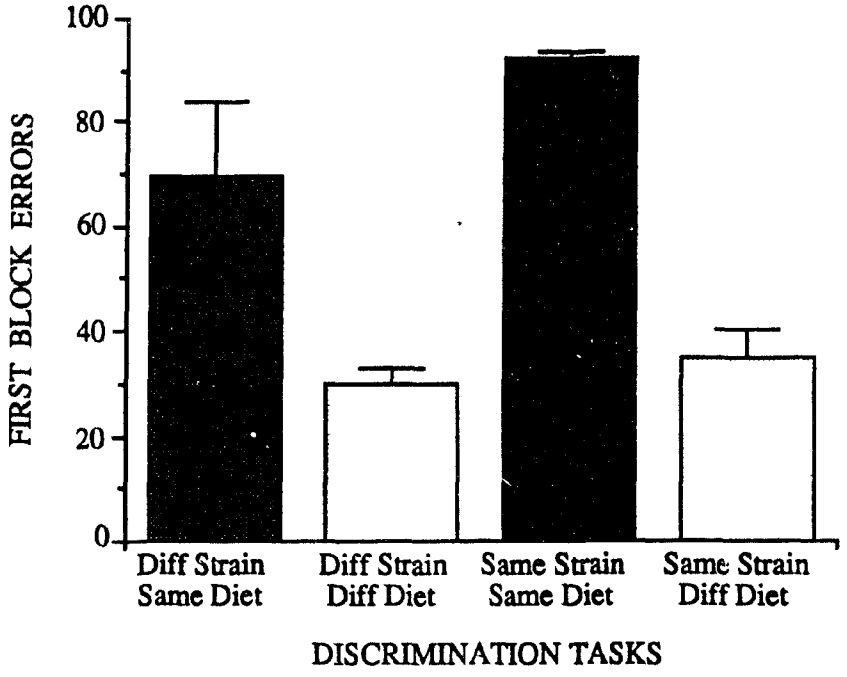


Figure 11.3. The mean (±sem) number of first block errors to reach a criterion of 85% correct for three consecutive sessions (or for a maximum of 10 sessions) on each of four urine odour discrimination tasks.

find it more difficult to remember a discrimination between the odours of mice which are genetically identical and maintained on the same diet, than when the mice differ by strain or diet.

DISCUSSION

The results of Experiment 8 indicate that, for rats, it is easier to discriminate between the urine odours of mice which differ in their diets than it is to discriminate between the urine odours of mice which differ at a single locus of the MHC (Figure 11.2).

The outcome of this experiment is contrary to the hypothesis that the MHC provides the most salient cues for the odours of individuality in mice and rats (Beauchamp et al., 1986; Brown et al., 1990). When rats were required to discriminate between urine samples from MHC congenic mice maintained on different diets (Task 2), they were able to remember the discrimination from session to session, indicating that this discrimination was based upon a constant odour cue. The subjects discriminating between the urines of genetically identical mice maintained on different diets (Task 4) were also able to find constant cues to discriminate, suggesting that dietary cues can provide a constant cue independent of whatever MHC genetic-based cues may be available in the urine.

Is there any evidence that rats attended to MHC cues in the urine odours? One subject met the criterion of 85 percent on the first block of trials over three sessions on Task 1 and one other subject reached a level of 85 percent on two non-consecutive sessions. Because the remaining two subjects did not find constant discriminable cues in the urines, the results were not significantly different from those of Task 3. It should be noted, however, that none of the subjects in Task 3 ever scored above 70 percent on the first block of any session. Thus, none of the rats learning to discriminate between genetically identical mice

raised on the same diet retained a memory for these odours. They could learn to make the discrimination within each session as shown in Figure 11.1C.

Mice can discriminate between the urine odours of MHC congenic mice of the BL6 and BM1 strains in a Y maze (Beauchamp et al., 1990). The ability of these mice to transfer training and respond to odours from unfamiliar mice of the same strain as the mice whose odours were previously rewarded, indicates that a difference in H-2 type provides a constant cue in the urine that can be used for individual recognition (Yamazaki et al., 1983). Thus, the inability of two of the four subjects learning Task 1 to find constant cues between the urine odours of congenic mice which differed at one locus of the MHC is puzzling.

One must conclude that, in this experiment, the rats learning Task 1 discriminated between odour pairs by using qualitative cues which were difficult to remember from day to day or that they based their discrimination upon quantitative differences in the urine samples, rather than qualitative differences influenced by the MHC. It is not known how the genetic differences which originate at the MHC influence odours of individuality (Boyse et al., 1983). As noted previously, H-2 type affects hormone metabolism (Ivanyi, 1978) and it is reasonable to expect that such metabolic variations would produce discriminable differences in the urine. As these quantitative differences could be expected to fluctuate from day to day, any discrimination based upon these cues would not be constant each day and the rats would need to reanalyze the components of the urine to find discriminable differences on every test session.

The mouse strains used in this experiment differed only at one MHC locus. Yamazaki et al. (1983) have found that mice trained in a Y-maze olfactometer took longer to learn to discriminate between the urine odours of two strains of MHC congenic mice which differ at one locus than it did to learn to discriminate between the urine odours of

mice which differ at three loci. Although rats are able to discriminate between the urine odours of mice which differ at one MHC locus (Beauchamp et al, 1990), it is possible that the difference in the two urine odours is only slight and that a greater genetic difference between the two mouse strains would produce a more salient difference in urine odour. Such a difference might provide cues which could be learned and remembered more easily. This hypothesis was tested in Experiment 9.

EXPERIMENT 9

IS IT EASIER TO DISCRIMINATE BETWEEN THE URINE ODOURS OF MHC CONGENIC MICE WHICH DIFFER AT THREE LOCI THAN AT ONE LOCUS?

To determine if congenic mouse strains that differ at three MHC loci would be easier to discriminate than strains that differ only at one MHC locus, I tested the ability of four rats from the original study which had not been previously tested on odours from BL6 mice to discriminate between samples of urine collected from BL6 and B6.AKR-H-2^k/Fla Eq (B6-H-2^k) mice, two strains which differ at three loci in the H-2K region. The procedure for the urine collection was the same as that described in Experiment 8.

The rats were tested in the operant olfactometer until they met the learning criterion of 85% correct on the first block of trials and a mean of 85% on the remaining nine blocks for three consecutive times or for a maximum of ten sessions. A comparison was made between the performance of these rats and the performance of the rats which had been trained to discriminate between urine odours from mice which differed only at one loci (Task 1 in Experiment 8). ANOVA revealed that there were no differences between the number of sessions to reach criterion [$F(1,6) = 1.0$, Table 2A], the number of errors to

Table 11-2. Number of sessions (A), number of errors to criterion (B), number of first block errors (C) required for the four subjects in each group to meet criterion (or for a maximum of ten sessions) in the discrimination of urine odours from MHC congenic mice which differed at three loci (BL6 and BM1) versus one locus (BL6 and B6-H-2^k).

A. Sessions to Criterion	Subject				
Task	1	2	3	4	Mean
3 loci	10	10	10	10	10
1 locus	10	10	10	6	9

B. Errors	Subject				
Task	1	2	3	4	
3 loci	452	860	532	429	568.25
1 locus	234	511	892	426	515.75

C. First Block Errors	Subject				
Task	1	2	3	4	
3 loci	79	98	74	83	83.5
1 locus	33	100	83	62	69.5

criterion [F (1,6 = .095, Table 11-2B] or the number of first block errors to criterion [F (1,6) = 1.01, Table 13-2C]. Consequently, the hypothesis that a genetic difference of only one locus at the MHC results in a more difficult odour discrimination for rats must be rejected.

The results of Experiment 9 eliminate the possibility that the discriminability of the urine samples from the MHC congenic mice on the same diet was influenced by the number of MHC loci by which the mice differed.

GENERAL DISCUSSION

How does one explain the result that the dietary cues were learned and remembered more readily than MHC-related cues? There are at least two hypotheses which should be considered:

1. The diet-based cue in the urine was more salient than the genetic-based cue. While it is unlikely that dietary cues could form the entire basis of odours of individuality, a number of studies with rodents have indicated that dietary cues exert a powerful influence upon the ability of animals to recognize other individuals. Adult male Long-Evans rats readily discriminate between the urine odours of identical mice on two different diets As shown in Experiment 7 (Chapter 10), rat pups are able to discriminate between the odours of their mothers and those of other lactating females only when the two are maintained on different diets (Leon, 1975). Adult female spiny mice prefer to retrieve pups born to mothers maintained on the same diet as themselves versus pups born to mothers on a different diet (Doane & Porter, 1978). Moreover, it appears that the influence of diet can be produced prenatally. Hepper (1988b) determined that, when rat pups had only prenatal experience with a particular diet, they subsequently showed a preference for that diet.

In Experiment 8, each mouse on diet P and diet H had exactly the same diet from day to day, thus, there was a consistent difference between the two diets. An odour produced by such a difference could be very salient. In the wild, it is unlikely that two mice would eat exactly the same different diet each day. If the mice had been fed on diets which differed randomly each day, a consistent dietary cue might not have been available and the genetic cue might have been attended to more readily.

2. Rats find it harder to discriminate genetic differences between mouse odours than between rat odours. Rats in an olfactometer are capable of using a constant cue to discriminate between the urine odours of congenic rats differing at one locus of the MHC (Schellinck et al., 1991). One of the four rats in the present study was able to remember the discrimination between mouse urine odours for three consecutive sessions, another scored 85 percent or more on every block of non-consecutive sessions and the other two rats could learn the discrimination within a session, they just could not remember it between sessions. Therefore, the poor performance of three of the subjects learning Task 1 could not be attributed to a lack of discriminable cues between the MHC congenic mice nor to an inability of rats to remember a constant cue based upon genetic differences.

It is possible, however, that the rats did not readily attend to genetic cues and remember these cues from session to session because these odours were from individuals of a different species. In a natural habitat, a rat's memory for mouse odours would not facilitate recognition of kin nor be relevant to mate selection. Thus, it may be that even though MHC-based odours of mice could be learned by rats within a test session in this operant situation, cues based upon such genetic differences may not be salient enough to be remembered between test sessions by individuals of another species. There is evidence that olfactory cues provided by dietary factors may provide more relevant information than species-typical cues. Spiny mouse pups prefer the maternal odours of outbred laboratory

mice on a familiar diet to the maternal odours of females of their own species on an unfamiliar diet (Porter & Doane, 1978). Porter et al. (1989) suggest that diet and genotype contribute additively to the individual odours of spiny mice.

In this experiment, changes in diet were more influential than genetic differences at the MHC in producing a discriminable odour. It is possible that the genetic difference was less salient because rats were learning to discriminate between the odours of mice rather than of other rats. To further clarify this hypothesis, Experiment 10 in Chapter 12 will examine the interaction of diet and genotype in MHC congenic rats so that I can study the response of rats to the odours produced by individuals of their own species.

CHAPTER 12

EXPERIMENT 10

WILL A DIET WHICH IS KNOWN TO ALTER GUT BACTERIA ELIMINATE URINE ODOURS OF INDIVIDUALITY OF RATS?

Evidence that a change in diet alters the nature of the gut bacteria and odour of the rat ingesting the diet was provided by Leon (1974) who maintained lactating female rats on a diet containing sucrose as the only carbohydrate. This particular diet alters the bacteria of the caecum, i.e., the pouch at the junction of the small and large intestines, because sucrose is completely absorbed in the small intestine. Consequently, bacterial growth is inhibited in the caecum because of a lack of an adequate growth medium. Leon determined that rat pups were no longer attracted to the anal excreta or caecal contents of mothers on this diet . He hypothesized that the faecal odours of mothers on the sucrose diet were not attractive to pups because this diet eliminated "the maternal odour" produced by lactating females on standard laboratory diets.

Although the effect of the Purina and Hagen diets used in Experiments 8 and 9 (Chapter 11) on the overall bacterial content of the gut was not determined, it seems likely that these diets altered the nature of the bacteria present. To further establish the relationship between bacteria and diet on the production of discriminable odours, a habituation-dishabituation task was used to examine the ability of rats to discriminate between the odours of MHC congenic rats maintained on Purina lab chow or on a Teklad test diet. The Teklad diet is the sucrose diet used by Leon (1974) and thus prevents the growth of caecal bacteria.

My previous experiments which assessed the importance of genetic and dietary cues in the production of odours of individuality (Chapters 10 & 11) could be criticized becausethe test animals, i.e., rats, were not of the same species as the odour donors, i.e. mice. Thus, the purpose of this experiment was twofold: (1) to determine if rats discriminate between the odours of other rats which differ genetically or which are on different diets; (2) to assess the influence of a diet which specifically alters caecal bacteria in eliminating odours of individuality. The habituation-dishabituation apparatus was used to test the discriminability of the urine odours.

METHOD

Subjects

Thirty-four male Sprague Dawley rats obtained from Charles River Canada (St. Constant, Que.) at a weight of 250-275 gm were used as subjects. They were individually housed, provided with Purina Lab Chow and water ad lib and maintained on a 12:12 reverse L:D cycle, with lights off at 9:00 a.m. The rats were tested when approximately three months of age.

Urine Donors

The urine donors in this experiment were male rats of the PVG and PVG.R1 strains obtained from Harlan Olac Ltd., Shaw's Farm, Blackthorn, England at six weeks of age (PVG: n = 8; PVG.R: n= 8). These strains of rats differ only at one locus of the class Ia genes of the MHC.

The rats were placed on a diet of Purina lab chow and four weeks later, their urine was collected for one month as described below. Their diet was then changed to Teklad fat sufficient test diet (TD 69446) and as suggested by Michael Leon (personal communication) urine collection was re-initiated after two weeks. The Purina diet had the following constituents (in g/Kg): 253 - crude protein; 48 - fat; 581 - carbohydrate; 10.2 - calcium and 8.4 - phosphorous. The Teklad diet consisted of the following: (in g/Kg): casein - 211; sucrose - 584.5; corn oil - 50.0; cellulose - 104.5; Mineral Mix - 40.0; Vitamin mix - 10.0.

The urine donors had free access to food and water except during the eight hour urine collection period when only water was available. Urine was collected by placing donors in a metabolism cage for eight hours per day during the dark phase of the light:dark cycle (L:D). A fecal bolus was collected from several urine donors before and after the change in diet and a colony count of gram negative and gram positive bacteria determined by the Dept. of Microbiology, Victoria General Hospital, Halifax, N.S. Contrary to expectations, the diet did not reduce the fecal bacteria count. Rather, it greatly increased the number of bacteria (colonies/ml), as shown in Table 12.1.

Apparatus and Procedure

The habituation-dishabituation apparatus and procedure as fully described in Chapter 6. was used in this experiment. Each subject received nine sequential two minute odour presentations with water presented for the first three trials, followed by three presentations of Urine Sample A and three presentations of Urine Sample B. The time spent rearing and sniffing each odour was recorded with a stopwatch by an observer blind to the different experimental conditions. All subjects were tested during the dark phase of the L:D cycle,

under dim white illumination and the test chamber was cleaned with a 70 % ethyl alcohol solution between subjects.

Subjects were tested with one of the following pairs of stimuli:

1) Two urine samples collected on different days from the same rat or from genetically identical rats maintained on Purina lab chow or two urine samples from the same rat or from genetically identical rats collected after their diet was changed to the Teklad test diet; two of the samples before and after the diet change were from each strain of MHC congenic rats (8 subjects tested).

2) Urine samples collected from rats of the two MHC congenic strains maintained on Purina rat chow (8 subjects tested); the order of presentation of samples was counterbalanced so that four of the subjects were presented with urine from PVG rats on Trial 4 and urine from PVG.R1 rats on Trial 7 and four were presented with PVG.R1 urine on Trial 4 and PVG urine on Trial 7.

3) urine samples from rats of the two MHC congenic strains maintained on the Teklad test diet (10 subjects tested); the order of presentation of samples was counter-balanced so that four of the subjects were presented with urine from PVG rats on Trial 4 and urine from PVG.R1 rats on Trial 7 and four were presented with PVG.R1 urine on Trial 4 and PVG urine on Trial 7.

4) urine samples from the same individual or individual rats of the same strain collected before and after their diet was changed (8 subjects tested); half of the samples were collected from PVG rats and half from PVG.R1 rats. Two of the samples from each strain were from the same donor before and after the diet change and two of the samples were from different donors before and after the diet change.

Statistical Analysis

A randomized block ANOVA was used to examine differences in the amount of time spent sniffing the odour samples over the nine trials. Newman-Keuls post hoc comparisons were used to determine which means differed from each other.

RESULTS AND DISCUSSION

In task 1, rats presented with different samples from the same rat or samples from genetically identical rats of either the PVG or PVG.R1 strains on the same diet did not discriminate between different individuals (Figure 12.1A). There were significant differences in the investigation times over the nine odour presentations [$F(8, 56) = 40.39$, $p < 0.001$] but only trial 4 differed significantly from any other trial ($p < 0.01$). Thus, subjects responded to the first urine sample presented, but did not respond differently to the second urine sample from the same rat or genetically identical rats on the same diet (trial 7).

In task 2, rats discriminated between urine samples from PVG and PVG.R1 rats when both strains of rats were maintained on Purina lab chow (Figure 12.1B). There were significant differences among the means for all eight subjects [$F(8, 56) = 47.78$, $p < 0.001$] and more time was spent sniffing the odour samples on trials 4 and 7 than on any other trials ($p < 0.01$). Subjects dishabituated when presented with a urine sample after the water presentations (trial 4), and also when presented with a second sample from a rat of a different strain on the same diet (trial 7).

In task 3, subjects were presented with urine samples from rats of the two different strains after their diet had been changed to the Teklad formula. There were significant

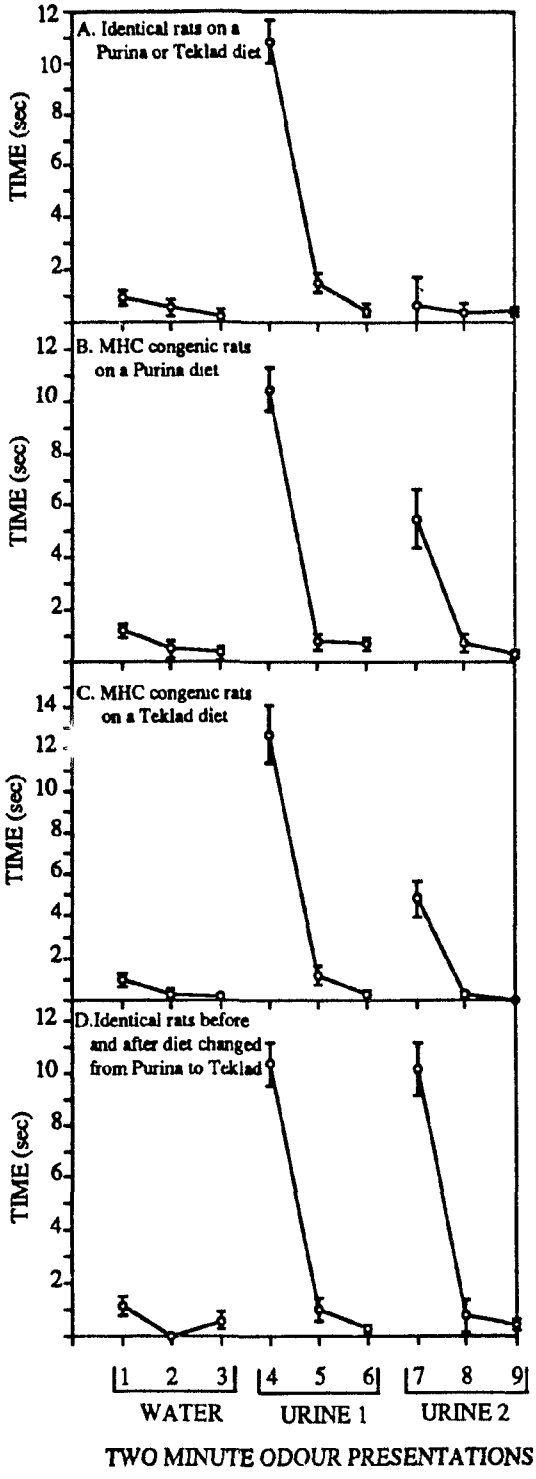


Figure 12.1. Mean (± sem) time (sec) spent investigating urine odours from (A) individual rats of the PVG or PVG.R1 strain maintained on either a Purina or a Teklad diet; (B) rats of the PVG and PVG.R1 strains maintained on a Purina diet; (C) rats of the PVG and PVG.R1 strains maintained on a Teklad diet and (D) individual rats of the PVG and PVG.R1 strains before and after their diet was changed from Purina to Teklad.

differences among the means for all seven subjects [$F(8, 48) = 58.27$, $p < 0.001$] and more time was spent sniffing the odour samples on trials 4 and 7 than on any other trials ($p < 0.01$). If the odours of individuality had been eliminated by the Teklad test diet, one would not have expected the two samples to be discriminable. Thus, it would appear that the change in diet did not eliminate their odours of individuality. As the bacterial analysis (Table 12.1) indicated that the bacteria were increased rather than eliminated, this result was not unexpected.

In task 4, subjects dishabituated when presented with samples collected from two genetically identical rats of the same strain before and after their diet had changed (Figure 12-1D). There were significant differences among the means for all eight subjects [$F(8, 56) = 62.86$, $p < 0.001$] and more time was spent sniffing the odour samples on trials 4 and 7 than on any other trials ($p < 0.01$).

Magnitude of Response on Trial 7. To determine if the increase in sniffing odours on Trial 7 was related to the type of urine sample introduced, I calculated the difference in sniffing time between trials 6 and 7 in each of the four tasks. An ANOVA revealed significant differences between groups [$F(3, 30) = 18.06$, $p < 0.001$] and a Newman-Keuls post hoc analysis indicated that the increase in sniffing time was significantly greater ($p < .01$) when the diet rather than the strain of individuals changed on trial 7 (Figure 12.2).

In general, these results indicate that the urine odours of an individual rat were altered but not eliminated by changing the rat's diet to one designed to prevent the growth of caecal bacteria. Furthermore, the urine odours of two individual rats of different strains could still be discriminated after the change to the Teklad diet. This provides further evidence that the removal of the caecal bacteria when rats were switched to the Teklad test diet was

Table 12.1. The total gram negative and gram positive bacteria in a random sample of rats maintained first on Purina rat chow and then on a Teklad test diet designed to eliminate caecal bacteria.

Subject		PURINA DIET	TEKLAD DIET
PVG101	Gram negative	1,800,000	7,400,000
	Gram positive	90,000,000	240,000,000
PVG.R152	Gram negative	6,000,000	33,000,000
	Gram positive	900,000	6,000,000
PVG100	Gram negative	40,000,000	400,000,000
	Gram positive	190,000,000	720,000,000

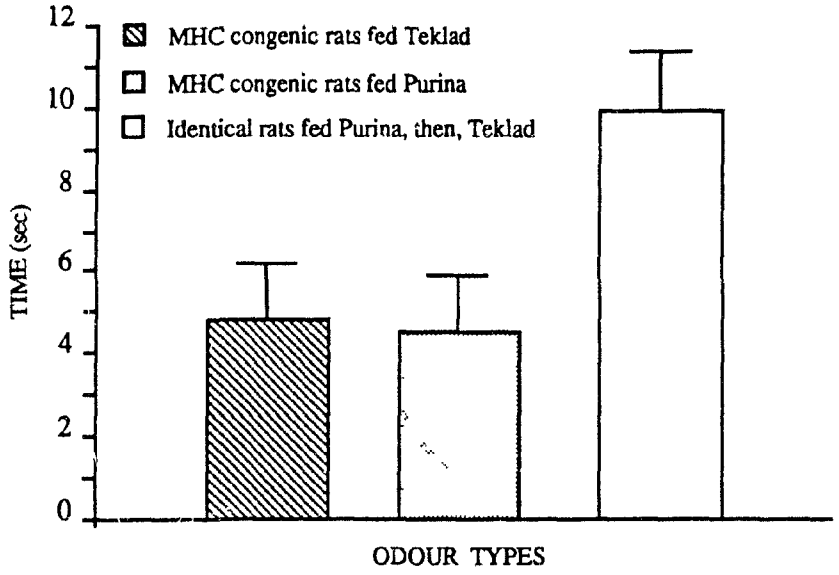


Figure 12.2. The mean (± sem) difference between the time (sec) spent investigating urine odours on trial 6 and trial 7 when the stimulus on trial 7 was: from a rat of a different strain on the same diet (Teklad) as the rat on trial 6 (▨); from a rat of a different strain on the same diet (Purina) as the rat on trial 6 (□) or from a rat genetically identical to the rat on trial 6 but on a different diet (□).

was not sufficient to eliminate the odours of individuality.

The response to the second odour was greater when an odour sample from an individual of the same strain on a different diet was presented on trial 7 rather than an individual of a different strain on the same diet. Consequently, one might conclude that changing the diet creates a more novel stimulus than does changing the strain. Together with the results of Experiments 8 and 9 in which rats found it easier to discriminate between the odours of genetically identical mice on different diets than MHC congenic strains of mice on the same diets (Chapter 11), this result suggests that dietary cues are more influential than genetically-based cues in determining odours of individuality. One theory of kin recognition is based upon the premise that individuals treat as kin any individuals which are found in the same spatial location as themselves. As one would anticipate that food sources would be similar within a specific location, degree of familiarity of dietary cues could also be partly responsible for recognition that appears to be based upon spatial distribution.

Although it has been shown that rats can discriminate between the odours of MHC congenic rats (Singh et al., 1987; Schellinck et al., 1991, Experiment 12), the ability of rats to generalize this learning to samples from unfamiliar individuals of the same congenic strains has not yet been demonstrated. Given the experimental results of this chapter in which diet cues seem to be more influential in providing discriminable differences in the odours of rats than the MHC cues, it would seem appropriate to determine if the urine odours of MHC congenic rats provide constant discriminable cues. In Chapter 13, I will use the operant-olfactometer to address this issue. In addition, if the subjects acquire the concept of MHC, I will determine if changing the diet of the odour donors disrupts the discrimination based upon cues provided by the MHC.

CHAPTER 13

WHAT ARE THE EFFECTS OF CHANGING DIET AND STRAIN CUES ON CONCEPT LEARNING IN RATS?

EXPERIMENT 11

ARE RATS ABLE TO LEARN THE CONCEPT OF MHC AS A SIGNAL OF INDIVIDUALITY AND IF SO WILL THIS CONCEPT BE DISRUPTED BY A CHANGE IN THE DIET OF THE MHC CONGENIC STRAINS?

To demonstrate that mice use constant cues to discriminate between the urine odours of MHC congenic mice, Yamazaki and his colleagues trained mice in a Y-maze with a number of odour samples from MHC congenic mice and then introduced random non-reinforced probe trials of samples from previously undiscriminated individuals of the same strains (Yamaguchi et al., 1981; Yamazaki et al., 1982). Since the mice responded to S+ probes as if they were the same as the odours used in the original training, it was concluded that the MHC provided constant discriminable cues which were discriminable in the urine.

It is likely that rats trained in an operant-olfactometer could also generalize their responses to samples from previously unknown individuals; however, in Experiments 5 and 6 of this thesis, I have demonstrated that the outcome of probe trials may be difficult to interpret. Consequently, to affirm that the "MHC concept" can be learned by rats as it can by mice, I used another procedure. Rats were trained to discriminate between urine samples from MHC congenic conspecifics; then in the middle of a training session, they

were given samples from novel individuals of the same strains on the same diet to assess their ability to transfer their training. To further determine the role of diet in influencing cues of individuality, I also evaluated the ability of these rats to generalize their learning of MHC congenic strains to the odours of these same individuals after their diet had been changed.

METHOD

Subjects

Six Long-Evans male rats purchased from Charles River Canada (St. Constant, Que.) at a weight of 250-275 gm were used as subjects. They were individually housed in 48 x 27 x 16 cm polycarbonate cages with stainless steel grid covers and woodchip bedding and maintained on a reversed 12:12 L:D cycle, with lights off at 9 a.m. Ten days prior to training, their water intake was restricted to 10-15 ml per day for the duration of the experiment.

Urine Donors

The 15 MHC congenic PVG and PVG.R1 donor rats used in Experiment 10 (Chapter 12) also provided the urine for this experiment. The urine had been kept frozen at - 20° C for use in the present experiment. As noted previously, the rats were placed on a diet of Purina lab chow and after two weeks, their urine was collected for one month as described below. Their diet was changed to the Teklad test diet (TD 69446) and after two weeks,

urine collection was re-initiated. More specific details of dietary components and urine collection procedures are found in Chapter 12.

The odours from the outbred Long-Evans rats were selected from a bank of donors from which urine samples had been collected and kept frozen at -20° C until required for use in odour discrimination experiments.

Apparatus and Procedure

Odour discrimination was evaluated in two computer-controlled operant olfactometers as described in Chapter 6. Following pretraining procedures with urine from outbred rats as the discriminable stimuli, the rats were trained to discriminate between urine odours from two strains of MHC congenic rats on a Purina diet. Odours from rats of one strain were designated as the S+ for three of the subjects and odours from rats of the other strain were designated as the S+ strain for the other three subjects.

To eliminate the possibility that discrimination was a result of extraneous differences resulting from changes in concentration or inadvertent contamination with feces or foodstuff, the samples collected from each individual on different days were first pooled into a large pool. Next, aliquots of samples from two-three individuals of the same strain were pooled to make up the test sample. Sufficient combinations of samples were made such that different stimuli were presented for each training session.

Once the subjects reached the discrimination criterion of 85 % correct on each block of trials in a session, they were switched to a 50 % partial reinforcement schedule for the S+ odours. As the S- odours were never reinforced, this essentially created a 25 % reinforcement schedule over all 200 trials. The presentation of S+ stimuli was such that a maximum of six non-reinforced S+ trials were presented in succession. As soon as the

criterion of 85% on each block had been re-established, the following pairs of test odours were introduced on Block 6 of one of the next five sessions:

(Task A): The same S+ and S- samples as discriminated on blocks 1-5 (same stimuli) were removed and immediately replaced to determine if the switching procedure caused any disruption.

(Task B): S+ and S- samples composed of novel combinations of urine pools from previously discriminated individuals of the same strains (i.e. known stimuli);

(Task C): S+ and S- samples composed of urine pools from previously undiscriminated individuals of the same strains (i.e. concept stimuli);

(Task D): S+ and S- samples composed of urine pools from previously discriminated individuals after their diet had been changed to the Teklad test diet (new diet stimuli);

(Task E): S+ and S- samples composed of urine pools from two previously undiscriminated outbred Long-Evans rats (unknown stimuli).

The first three pairs of odours were presented in a quasi-random order such that only two of the six subjects received the same type of odour pairs on the same day. All subjects were tested with the unknowns on the fourth day of testing and the new diet stimuli on the fifth day of testing.

Prior to the start of block 6, the odour pairs were introduced by exchanging the stimulus jars used on blocks 1-5 with stimulus jars containing the test odours. The S+ odour was not reinforced for the first five presentations on block 6, for any of the odour pairs.

Statistical Analysis

A repeated measures ANOVA was used to compare the difference in the number of errors between blocks 5 and 6 among the five tasks. Newman-Keuls post hoc comparisons were used to determine which means differed from each other.

RESULTS

The process of removing the same samples in mid-session and replacing them immediately did not disrupt performance (Task A). There were no significant difference in the number of errors on the two blocks ($t(5) = 1.00$; $p > .1$). Subsequently, this control group was not included in further analysis.

An ANOVA revealed significant differences in the number of errors between blocks 5 and 6 among the different tasks [$F(3, 15) = 11.5$, $p < .001$], (Figure 13.1). Post hoc analysis indicated that the performance did not differ when the newly introduced samples were from previously discriminated individuals of the same strains (Task B) or from previously undiscriminated individuals of the same strains (Task C). Performance on these two tasks differed ($p. < .01$) from the tasks in which the introduced samples were collected from the previously discriminated strains on a new diet (Task D) and for samples from unknown outbred rats (Task E). The latter two did not differ from each other. Figure 13.2 shows the learning curves for a representative subject in tasks A to E.

These results indicate that the rats were able to learn the concept of MHC as demonstrated by their ability to correctly generalize their original training to new samples from previously undiscriminated individuals without a significant drop in performance. Nonetheless, a change in diet disrupted the perception of these cues to such an extent that

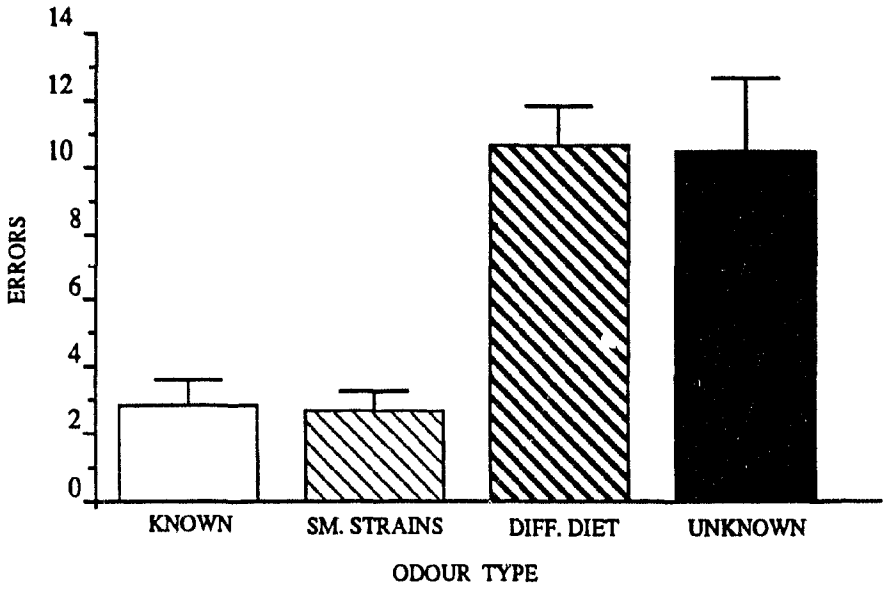


Figure 13.1. The mean (± sem) difference between the number of errors on blocks five and six for six rats after the discriminative stimuli (urine odours from MHC congenic rats on the same diet) were replaced with either known samples, samples from individuals of the same strains, samples from the same strains fed different diets or samples from unknown outbred rats.

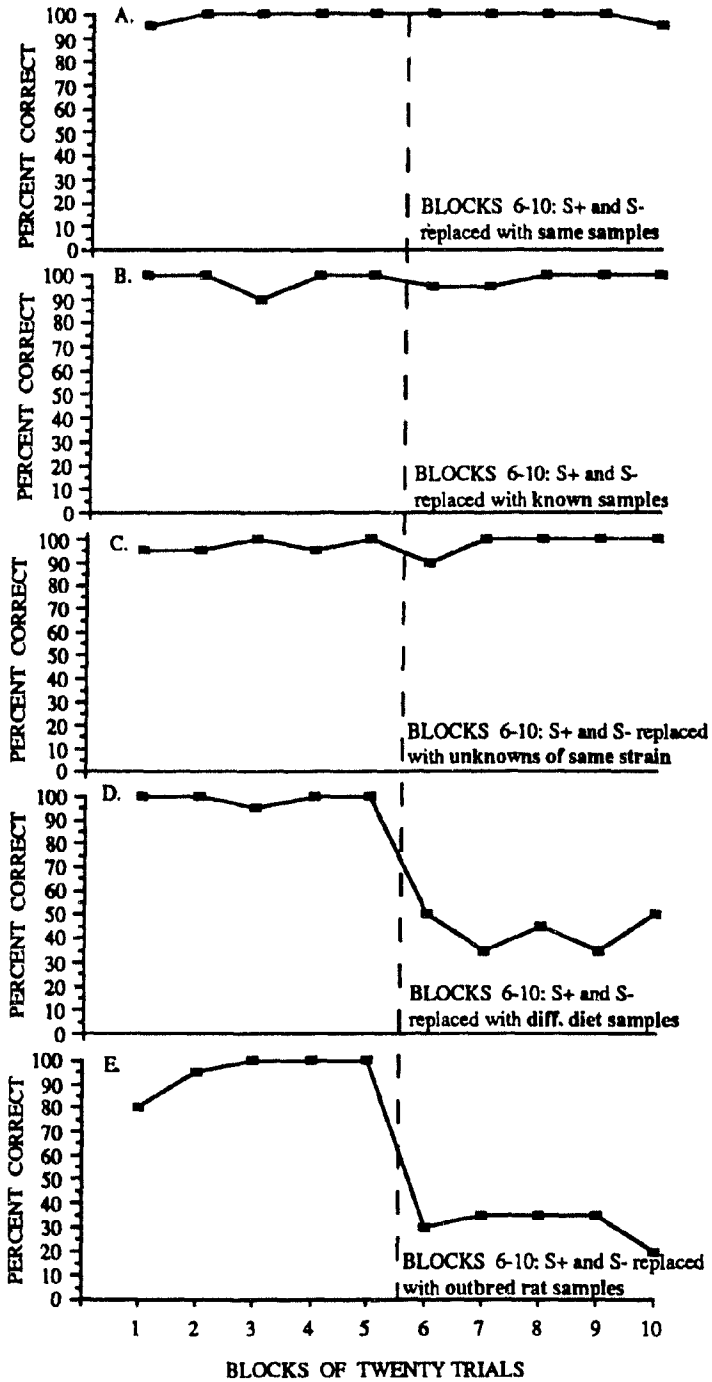


Figure 13.2. The learning curves of one rat (Slash) in the discrimination of urine odours from MHC congenic rats on Purina diet. On block 6 the S+ and S- stimuli were replaced with the following: A. the same samples; B. previously discriminated samples; C. previously undiscriminated samples of the same strains; D. samples from the same strains on different diets; E. samples from previously undiscriminated outbred rats.

the odours were found to be as different as odours from previously undiscriminated outbred rats.

EXPERIMENT 12

WILL THE ABILITY OF RATS TO DISCRIMINATE BETWEEN THE URINE ODOURS OF GENETICALLY IDENTICAL RATS ON DIFFERENT DIETS BE DISRUPTED BY CHANGING THE STRAIN OF RATS BUT NOT THEIR DIET?

Using the paradigm established in Experiment 11, it should be possible to determine if the ability of rats to discriminate between the urine odours of genetically identical rats on two different diets is disrupted when the urine odours are switched to different strains of rats on the same diets. In this way, the relative influence of dietary and MHC cues could be assessed. If the performance of the rats is disrupted when the strain of the odour donor rats is changed, this would provide evidence that cues provided by the MHC influence the discriminablity of odours.

METHOD

Subjects

Seven Long-Evans male rats were purchased from Charles River Portage (Portage, Mich.) at a weight of 250-275 gm. Their housing conditions and water restriction schedules were the same as those of the subjects in Experiment 11.

Urine Donors

The 15 rats used in Experiment 11 also provided the urine odours for this experiment. The odours from the MHC congenic mice which were used as unknown stimuli had previously been collected for use in the experiments described in Chapter 11.

Apparatus and Procedure

The odour discrimination tasks were assessed in two computer-controlled operant olfactometers as described in Chapter 6. Following pretraining procedures with urine from outbred rats as the discriminable stimuli, the rats were trained to discriminate between urine odours from genetically identical rats on two different diets, Purina lab chow and the Teklad test diet. The percent of nutrients in each diet was described in Chapter 12.

Four of the subjects were required to discriminate between genetically identical rats of the PVG strain on the two different diets. Donors maintained on Purina lab chow provided the S+ stimuli for two subjects; the S- stimuli were collected from donors maintained on Teklad test diet. The valence of the stimuli was reversed for the other two subjects. The remaining three subjects were required to discriminate between genetically identical rats of the PVG.R1 strain on the two different diets. For two subjects, the S+ stimuli was from donors eating a Purina diet and the S- from donors eating a Teklad diet. The S+ and S- odours were reversed for the third rat.

Once the subjects reached the criterion of 85 % correct on each block of trials in a session, they were switched to a 50 % partial reinforcement schedule for the S+ odours with a maximum of six non-reinforced S+ trials presented in succession. As soon as the

above criterion had been re-established, the following test pairs of odours were introduced on Block 6 of one of the next four sessions:

(Task A): The same S+ and S- samples as discriminated on blocks 1-5 (same stimuli);

(Task B): S+ and S- samples composed of novel combinations of urine pools from previously discriminated rats of the same strain on different diets (i.e., known stimuli);

(Task C): S+ and S- samples composed of urine pools from previously undiscriminated genetically identical individuals of a different strain (strain stimuli). These S+ and S- donors had been maintained on the same diets as the S+ and S- donors that each subject had previously learned to discriminate. The donors of these stimuli were MHC congenic to the previous donors. Thus, rats trained with PVG donors were given odours from PVG.R1 donors and vice-versa.

(Task D): S+ and S- samples composed of urine pools from two previously undiscriminated MHC congenic mice maintained on Hagen hamster diet, (i.e., unknown stimuli);

All four odour pairs were presented in a quasi-random order such that no more than two individuals were tested on the same types of odours in the same session. The odours were introduced by exchanging the stimulus jars as described in Experiment 11. As in the previous experiment, the S+ odour was not reinforced for the first five presentations on block 6, for any of the odour pairs.

Statistical Analysis

A repeated measures ANOVA was used to compare the difference in the number of errors between blocks 6 and 5 among the four tasks. Newman-Keuls post hoc comparisons were used to test for differences among means.

RESULTS

The process of switching the same samples in mid-session and replacing them immediately did not disrupt performance between blocks 5 and 6 as none of the subjects made any more errors on block 6 than on block 5 (Task A). Subsequently, this control group was not included in further analysis.

An ANOVA revealed significant differences in the number of errors between blocks 5 and 6 among the different tasks [$F(2, 12) = 330.9$, $p<.001$], (Figure 13.3). Post hoc analysis indicated that the subjects' performance did not differ when the newly introduced samples were from novel pools of previously discriminated individuals (Task B) or from previously undiscriminated individuals of a different strain on the different diets learned previously (Task C). Performance dropped significantly on block 6 however, when samples from unknown MHC congenic mice on an unknown diet were introduced (Task D) ($p.<.01$). The learning curves of a representative animal illustrate these results (Figure 13.4).

Thus, after the rats learned to discriminate between the urine odours of genetically identical rats on different diets, they were able to transfer this ability to discriminate between samples from genetically identical individuals of a different strain without

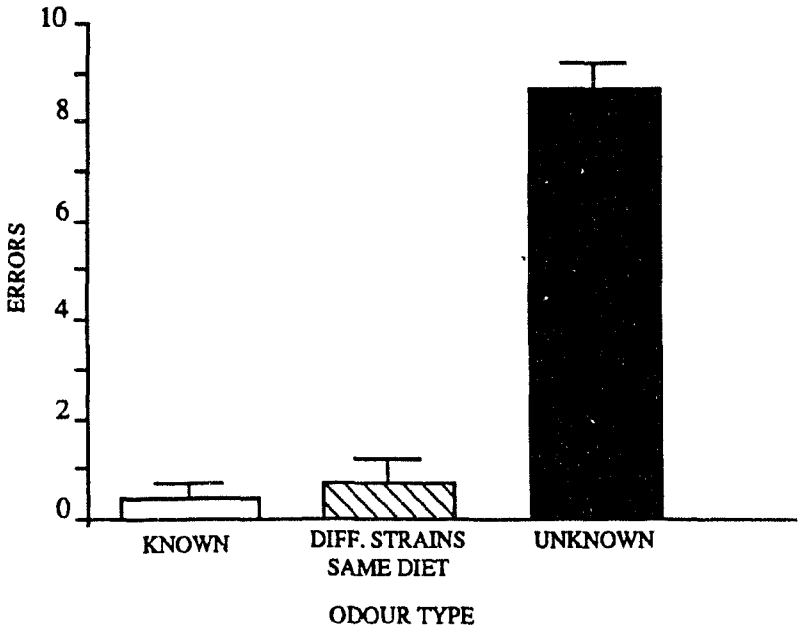


Figure 13.3. The mean (± sem) difference between the number of errors on blocks five and six for five rats after the discriminative stimuli (urine odours from genetically identical rats on different diets) were replaced with known samples, samples from individuals of a different strain on the same diets or samples from unknown MHC congenic mice on an unknown diet.

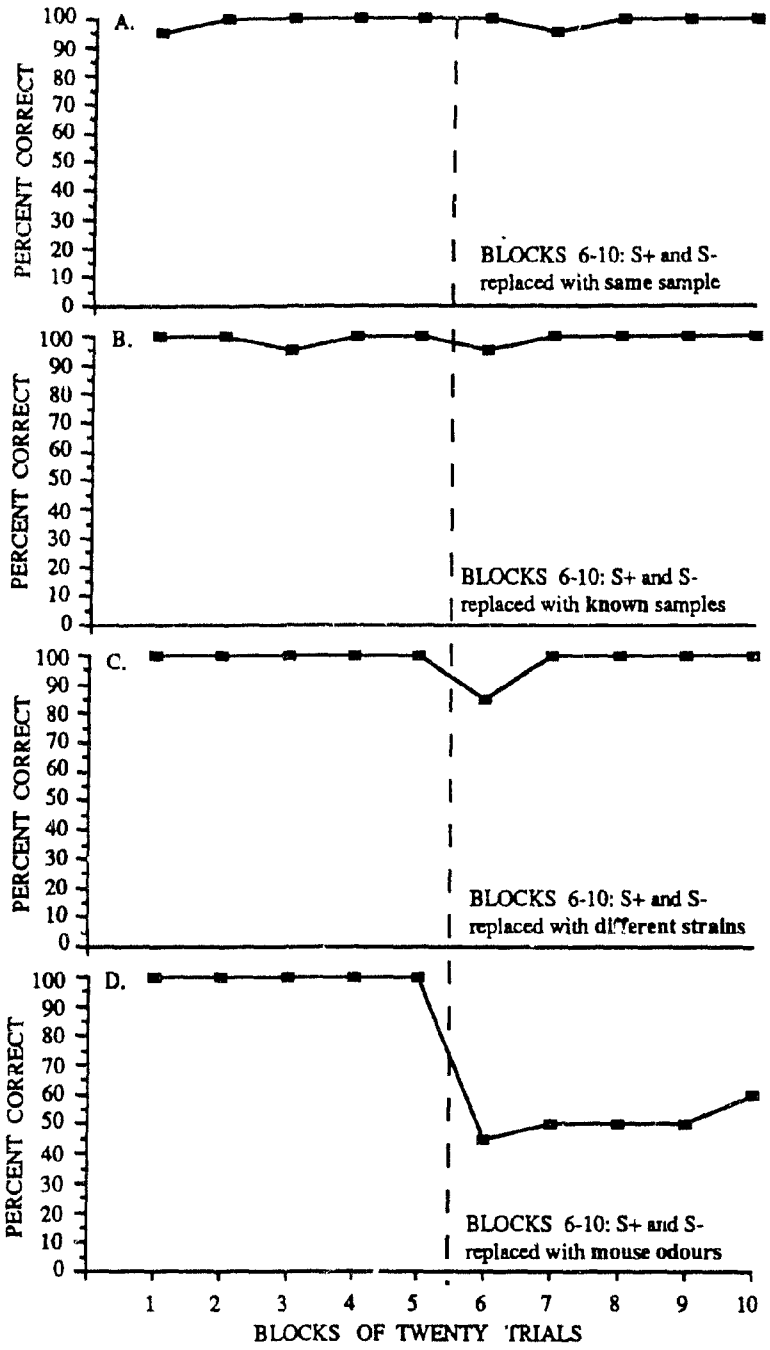


Figure 13.4. The learning curves of one rat (Wolfblass) in the discrimination of urine odours from genetically identical rats on different diets (Purina & Teklad). On block 6 of the session the S+ and S- stimuli were replaced with the following: A. the same samples; B. previously discriminated samples; C. samples from the same different strains on the same diets; D. samples from previously undiscriminated MHC congenic mice on an unknown diet (Hagen).

disruption as long as the diet cues remained the same. This result demonstrates that ,in this context ,the introduction of strain differences will not disrupt a discrimination based upon dietary cues.

DISCUSSION

The ability of rats which have learned to discriminate between the urine odours of MHC congenic rats to transfer this training to samples from previously undiscriminated individuals of the same strains without disruption of their performance has provided incontrovertible evidence that differences at the MHC can generate constant odour cues in rats.

While the results of my previous experiments (Schellinck et al.; 1991, Chapter 12) had shown that the odours of MHC congenic animals provided discriminable cues, they did not eliminate the possibility that the differences were the result of factors unrelated to the MHC. In this experiment, however, a comparison between the subjects' perception of known and unknown MHC congenic odours revealed that the two types of odours were not significantly different (Figure 13.1). These results also corroborate the similar findings of Yamazaki and his colleagues in which mice have been found to transfer training from a learned discrimination between MHC congenic mice in a Y-maze and respond to probe trials from unknown mice of the same congenic strains.

Despite this ability to transfer training from known to unknown MHC congenic conspecifics, other results of this experiment call into question the relative importance of these odour cues in the production of odours of individuality. A change in diet disrupted the perception of the MHC cues to the extent that the odours of individuals of the same

strains were found to be as different as odours from previously undiscriminated outbred rats (Figure 13.1).

Moreover, the introduction of strain differences did not disrupt a discrimination based upon dietary cues (Figure 13.3). This supports the finding that differences in diet provide a more salient stimulus than do the the odours of MHC congenics as measured by a greater amount of dishabituation in a dishabituation-habituation task (Chapter 12: Figure 12.2). These results reflect the significant effect of dietary cues in producing discriminable differences in urine odours and also indicate that, at least, in the conditions present in these experiments, such cues take precedence over genetic cues.

The specific aspects of the two diets which created the discriminable differences in urine odours were not determined. Both quantitative and qualitative differences in gut bacteria resulting from differences in the Purina and Teklad diets may have been responsible for such an effect. While the sucrose content of the Teklad diet may eliminate production of caecal bacteria as hypothesized by Leon (1974), it appears to provide a more effective overall substrate for the growth of gram negative and gram positive bacteria (Table 12.1). This general increase in bacteria could have subsequently provided quantitative changes in urinary volatiles, thus, providing discriminable differences in urine odours. Moreover, as the metabolism of different carbohydrates by bacteria also results in different end products (Dawes, 1992), it seems likely that qualitative differences in volatile urine products were also influential in providing discriminable odour cues.

Genetic differences in odours have been shown to provide discriminable cues in mate choice and communal nesting patterns (Egid & Brown, 1989; Potts et al., 1991; Manning et al., 1992). The next step in the investigation of the influence of dietary factors in the production of odours of individuality is to assess their role in such contexts as these. Moreover, it would be appropriate to use diets which are only somewhat different from

each other and also more similar to those encountered by rodents in their natural habitat. The quantitative differences in bacteria produced by the diets used here may have created such a salient discrimination that any influence a change in strain could have produced was ignored.

Taken together, the results of these two experiments have shown that both genetic and dietary cues play a role in the production of odours of individuality. While it seems likely that both factors contribute to such an odour, how they interact has yet to be determined.

CHAPTER 14

SUMMARY AND CONCLUSIONS

The 12 experiments reported in Chapters 7 to 13 of this thesis examined the role of bacteria, dietary cues or the MHC in contributing to odours of individuality in rats and mice. In this chapter, the questions asked will be reiterated, the most pertinent experimental findings summarized and the results discussed in terms of the hypothesis that the MHC and diet interact to determine the nature of gut bacteria, thus, controlling the nature of volatiles present in the urine. This chapter concludes with the presentation of a model which delineates the factors which may contribute to the urine odour profile of an individual.

14.1. The Questions

1. Are the urine odours of individuality of germfree mice discriminated by rats in an operant-olfactometer? (Experiment 1, Chapter 7)

Rats tested in an operant-olfactometer were able to discriminate between the urine odours of MHC congenic mice raised in germfree conditions as well as they were able to discriminate between the urine odours of mice raised in conventional housing. This result was in agreement with the findings of Yamazaki et al. (1990) which determined that mice trained in a Y-maze could discriminate between the urine odours of MHC congenic mice raised in germfree conditions. Thus, it provided further evidence that bacteria may not be necessary for the production of odours of individuality in mice.

2. Are the urine odours of individuality of germfree mice discriminated by rats in a habituation-dishabituation task? (Experiment 2, Chapter 7)

When tested in a habituation-dishabituation test, rats did not discriminate between the urine odours of MHC congenic mice raised in germfree conditions whereas they did discriminate between the urine odours of mice raised in conventional housing. This result confirms the report of Singh et al. (1990) which indicated that the urine odours of MHC congenic rats which were raised in germfree conditions were not discriminated by rats in a habituation-dishabituation task. While these findings contradict those of Experiment 1, they do provide the first indication that the absence of bacteria does alter a rat's perception of odours of individuality in mice.

The results of Experiments 1 and 2 together with those of Singh et al. (1990) and Yamazaki et al. (1990) would suggest that methodological factors are contributing tc the outcome of the discrimination experiments, i.e., odours of germfree individuals are discriminated in an operant task but not in a habituation-dishabituation task. Simply put, the results of the operant task reflect what the rat can be trained to do and the habituation-dishabituation task demonstrates what he will naturally do.

It is my opinion that, in the operant-olfactometer task, the interaction between the extraordinary ability of rodents to learn to discriminate between olfactory stimuli, the extended number of presentations of the odour stimuli and the motivation provided by the water-restriction schedule have created a procedure where odours which differ minimally can be discriminated. In contrast, in the habituation-dishabituation task, the subjects make their decision on the basis of a few sniffs, are not externally motivated to find differences between the samples and may, therefore, only pay attention to relevant differences.

There is evidence from other experiments which supports this viewpoint. Rats consistently dishabituated when presented with odours from individuals of different strains and have consistently responded non-differentially to the odours from genetically identical individuals (Brown et al., 1987; Singh et al., 1987; Brown et al., 1989). Thus, while one would not generally consider the absence of an effect proof of anything, in this context, a lack of dishabituation has provided a reliable indication that odours are not discriminated. Conversely, rats tested in the operant-olfactometer were able to discriminate between the odours of genetically identical rats and mice (Schellinck et al., 1991, Chapter 11 of this thesis), a strong indication that they can discriminate odours based upon undefined factors.

Based upon these latter results, as well as those of Experiments 1 and 2, I would also hypothesize that in the absence of bacteria, differences at the MHC are still minimally expressed and can be detected in an operant procedure; however, they are not sufficiently defined to be attended to in the habituation-dishabituation task. When bacteria are present, the differences in odours which are controlled by the MHC become more salient and are readily discriminated in the habituation-dishabituation procedure.

3. Are the urine odours of individuality of rats removed by elimination of gram negative or gram positive gut bacteria? (Experiments 3 & 4, Chapter 8)

Rats first presented with a urine sample of an individual rat before its gram negative bacteria were removed dishabituated when presented with a second sample from the same individual rat after its gram negative or gram positive bacteria were removed. Thus, it appears that the odours of an individual were altered by the removal of its gram negative or

gram positive bacteria; a finding which confirms the importance of bacteria in the process of identifying an individual.

Rats also discriminated between the odours of two individuals after the removal of their gram negative or gram positive bacteria despite the fact that massive numbers of their bacteria had been destroyed. Thus, one could also conclude that selectively removing bacteria was not a sufficient manipulation to eliminate urine odours of individuality in rats.

4. Will rats be able to detect changes in urine odours of individuality in the olfactometer after selective removal of bacteria (Experiments 5 & 6, Chapter 9)

Rats trained in an operant-olfactometer to discriminate between the urine odours of two individuals prior to the removal of their gram negative or gram positive bacteria did not respond significantly more to probe trials from these same rats after they had their gram negative or gram positive bacteria removed than tio odours from unknown rats; thus, confirming that the urine odours of an individual rat are altered by the selective depletion of gut bacteria. Nonetheless, in a follow-up experiment, it was determined that the odours of these same two individuals which had either their gram negative or gram positive bacteria could be discriminated with relative ease compared with the rats ability to discriminate between the odours of two unknown individuals. I concluded from this latter finding that the odours still retained some discriminable components which had been in the urine prior to the removal of the gram negative or gram positive bacteria. The rats had learned and remembered these cues during the training phase of the experiment and thus, in the relearning task, the odours were easily identifiable.

The ease with which the rats learned to discriminate between the odours of the donors after their bacteria were removed contradicts the premise that a lack of response to a probe trial is an indication that the probe odour is not the same as the training odour. Consequently, the results of this experiment emphasize that definitive conclusions about the nature of the information contained in a probe odour should not be drawn without further investigation of the subjects knowledge about the odours. This may be particularly true when assessing the discriminability of cues contained in such a complex mixture as urine.

5. Are rats able to discriminate between the urine odours of genetically identical mice maintained on different diets? (Experiment 7, Chapter 10)

Rats tested in a habituation-dishabituation test are able to discriminate between the urine odours of genetically identical mice which have been maintained on two different diets. As the diet of an individual is not likely to remain constant, it does not seem sensible to suggest that dietary cues could be solely responsible for individual odours used in kin and individual recognition. Nonetheless, the results of this experiment indicate that the presence of dietary cues contributes to an individual's odour profile. One could hypothesize that bacteria are responsible for the production of volatile end-products in the urine odours because of an interaction between the MHC and dietary cues. The MHC controls what bacteria are present (Howard, 1977) and changes in diet influence the quantity of each specific strain of bacteria.

In this experiment in which qualitative cues were not available as the mice were of the same MHC type, the rats could have used quantitative cues in making their discrimination. Rats have been shown to be very efficient at discriminating between different concentrations of the same compound (Slotnick & Ptak, 1977). For example a .01

concentration of amyl acetate could be discriminated from a .009 concentration . Thus, it seems likely that small quantitative differences in the volatile end products of bacteria could have been discriminable in this experiment.

6. Do dietary and genetic cues interact in the production of urine odours of individuality of MHC congenic mice? (Experiments 8 and 9, Chapter 11)

Rats found it easier to discriminate between the urine odours of genetically identical mice which differed in their diets than between the urine odours of MHC congenic mice on the same diet. The odour donors in this experiment only differed at one locus of the MHC; however, this could not account for the inability of the rats to perform well as the discrimination of urine odours from mice which differed at three loci was no easier. Two alternative hypotheses arise from this finding: either the diet-based cue is more salient than the genetic cue or rats find it harder to discriminate genetic differences between mouse odours than between rat odours.

7. Will a diet which is known to alter gut bacteria eliminate the urine odours of individuality of rats? (Experiment 10, Chapter 12)

The response of rats tested in a habituation-dishabituation task indicated that the urine odours of an individual rat were altered by a test diet designed to prevent the growth of caecal bacteria. Nonetheless, the urine odours of two individual rats of different strains could still be discriminated after the change in diet. Thus, the alteration in gut bacteria did not eliminate the odours of individual rats. This result was not unexpected as the fecal analyses from samples taken before and after the change indicated that the number of both

gram negative and gram positive bacteria had multiplied tenfold upon ingestion of the test diet.

It was clearly not the purpose of this experiment to test Leon's (1974) hypothesis that caecal bacteria are necessary for the production of maternal pheromone and that the elimination of such bacteria also eliminates the maternal odour. Leon based his hypothesis upon the theory that bacteria would be reduced in the caecum when there was no carbohydrate present for them to metabolize but he did not actually measure the change in bacteria (Michael Leon, personal communication, July, 1994). The fecal bacteria count only provide a general picture of the bacteria present in the caecum and large intestine. Nonetheless, my results indicate that the Teklad test diet increased rather than decreased the numbers of both gram positive and gram negative bacteria.

One would hypothesize that such a change may increase the odours present in the feces rather than remove them as Leon (1974) suggests. Consequently, the maternal pheromone may have been masked rather than eliminated by the change in diet. Despite the obvious differences between odour donors such as sex and strain in our experiments, my findings indicate that Leon's maternal pheromone hypothesis should be re-evaluated.

8. Are rats able to learn the concept of MHC as a signal of individuality and will this concept be disrupted by a change in the diet of rats? (Experiment 11, Chapter 13)

Rats trained in an olfactometer to discriminate between the urine odours of specific MHC congenic rats were able to generalize this training to odours from previously undiscriminated rats of the same strains. Such a result demonstrates that the concept of MHC as a source of a constant odour in rats can be learned. This finding provides support

for the results of Yamazaki et al. (1982; 1983) which showed that mice trained in a Y-maze could transfer their discrimination training to samples from unknown MHC congenic mice of the same strains learned previously.

When odours which had been collected from the original donors after their diet had changed were presented to the test rats in mid-session, the ability of rats to maintain their level of performance on the discrimination task was severely disrupted. The odours were as difficult to discriminate as odours from previously undiscriminated outbred rats. This result and those of Experiments 8 and 10 confirm that rats attend to cues made available by a change in diet. Moreover, the fecal bacteria analysis indicated that the number of bacteria increased dramatically (Table 12.1 pg. 248) after the change in diet, providing further evidence that dietary factors are associated with quantitative changes in the volatile end-products in the urine samples.

9. Will the ability of rats to discriminate between the urine odours of genetically identical rats on different diets be disrupted by changing the strain of rats but not their diet? (Experiment 12, Chapter 13)

The performance of rats which were trained to discriminate between the urine odours of genetically identical rats on different diets was not disrupted by switching the samples in midsession to samples from a strain of rats congenic to those first learned when the diet cues remained the same. Thus, it appears that the qualitative changes produced by the MHC cues may be ignored or overshadowed if the differences produced by dietary cues are particularly salient.

Nonetheless, it would be injudicious to conclude from this result that genetic cues are not of primary importance in the production of odours of individuality. In this experiment,

as in the other experiments in this thesis which assessed the relative discriminability of diet and the MHC, it is likely that the cues provided by the two diets were quite dissimilar. If dietary cues were only slightly different and inconsistent as would more likely be the situation in an animal's natural habitat, it is plausible that the influence of the MHC cue would also be perceived by a discriminating individual.

This hypothesis could be further investigated by manipulating the degree of difference in diet cues and assessing the response of rats in the paradigm used in this experiment. If changing the MHC type disrupted a discrimination based upon diet cues and if changing the diet cues affected the discrimination of MHC congenic strains, the hypothesis would be substantiated. Such a result would also confirm the hypothesis of Porter et al. (1989) that diet and genetic cues contribute additively to odour-based recognition signatures in spiny mice.

14.2. Origins of an individual odour profile

Overall, the experimental results of this thesis indicate that urine odours of individuality in rats and mice are influenced by dietary factors and genetic differences at the MHC. The presence of bacteria appears to be necessary for the appropriate expression of both of these cues. Nonetheless, many other factors such as the presence of disease, social status in males and reproductive status in females also produce discriminable odour cues. Consequently their contribution must be also be considered when determining the constituents of an individual's odour profile. I have developed a preliminary model which presents the genetic and environmental factors which contribute to such an odour profile (Figure 14-1). The purpose of the model is not to judge which theory regarding the

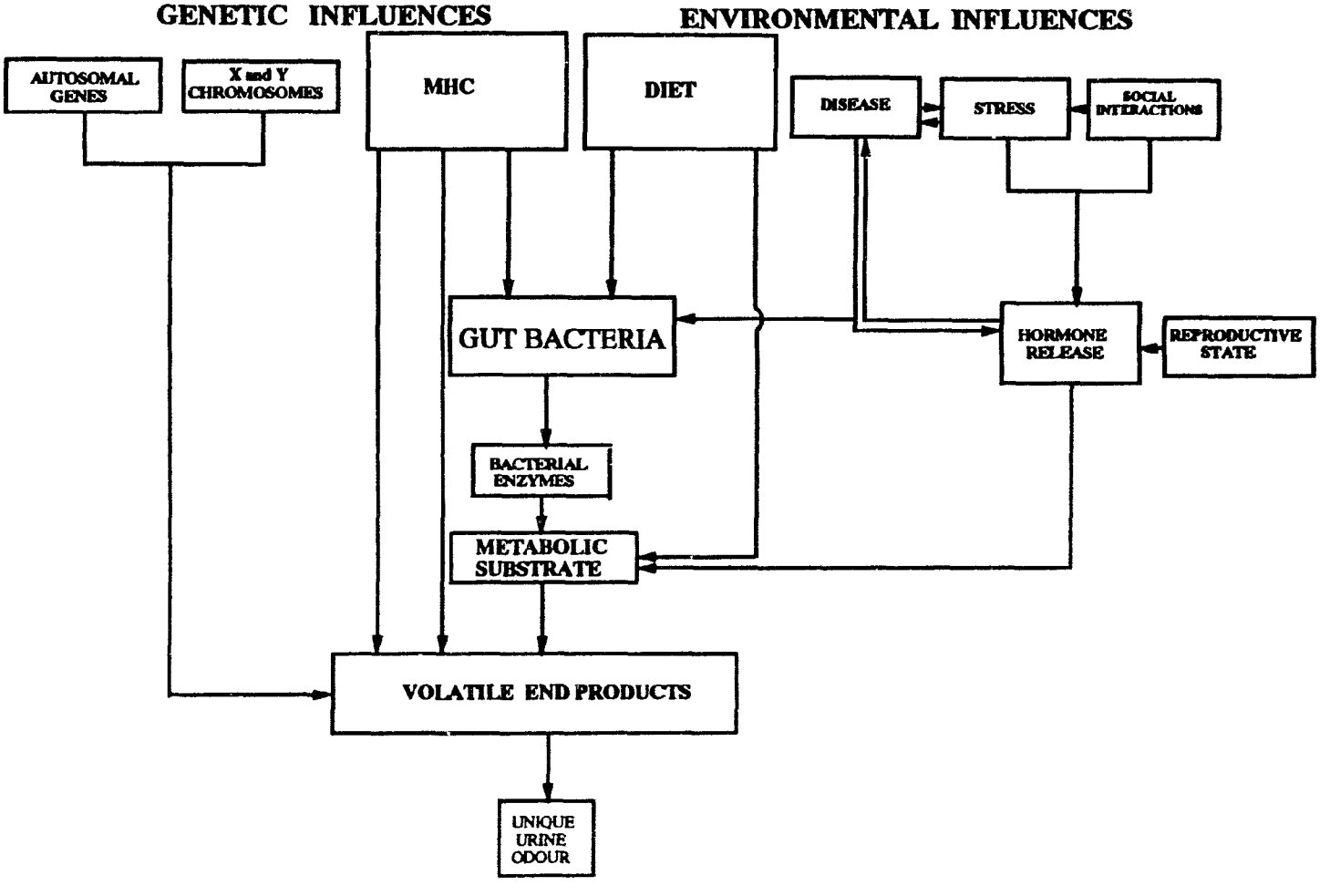


Figure 14-1. The Schellinck model of how the interactions among the MHC, bacteria, diet and other genetic, environmental and physiological factors are involved in the production of urinary odours of individuality in rats and mice.

production of odours of individuality is most valid but rather to illustrate the options available so that further research may take each of these factors into account.

14.2.1. MHC

Many experiments , including several in this thesis ,have demonstrated that the MHC contributes to urine odours of individuality. Unfortunately, the way in which this cue is expressed in the urine is unknown. Some investigators interpret the experimental evidence to mean that the presence of bacteria is necessary for optimal expression of MHC-driven production of odours whereas others do not accept this hypothesis. Those who believe in the bacteria hypothesis can so far rely only on theoretical arguments to explain the mechanism by which the MHC and bacteria interact (Howard, 1977). Nonetheless, those who are skeptical have not yet provided any definitive alternate explanation (Yamazaki et al., 1990).

Recent experiments which have revealed the connection between MHC-associated genes and growth and reproduction provide a direction for future research which might also reveal the link between MHC and odour production (Warner et al., 1987. 1991; Gill, 1992). Although the physiological mechanism by which this influence could be expressed has not been investigated, genes of the MHC are known to alter steroid hormones (White, New & Dupont, 1986) and to influence the length of estrous cycles (Lerner, Anderson, Harrison, Walford & Finch, 1992).

14.2.2. Other genes

Genetic factors other than the MHC also contribute to the production of odours of individuality. Odours of mice which differ in their non-MHC or background genes are discriminable (Beauchamp et al., 1990) as are the odours of mice which differ in their X and Y chromosomes and rats which differ in their Y chromosomes (Schellinck et al., 1993; Monahan et al., 1993). The way in which characteristics of autosomal or X and Y chromosomes are translated into discriminable urine differences has not been assessed.

14.2.3. Diet

In this thesis, changes in diet have been shown to alter the urine odours of rats and mice. In Experiment 10, quantitative changes in bacteria were found to be associated with a change in diet. This result confirms the many reports in the literature which have demonstrated that bacterial metabolism is dependent upon a dietary substrate and that the end-products of this metabolism are excreted in the urine. I have hypothesized that dietary components cause quantitative changes in the types of bacteria which have been previously selected for by the MHC. The first step in the investigation of this hypothesis would be to demonstrate that the MHC is responsible for qualitative changes in bacteria. If changes in the types of bacteria present are not found in MHC congenic strains, then the role of the MHC in must be re-evaluated.

14.2.4. Disease

The presence of disease may influence the odour of an individual via several routes. Depending upon the metabolic activities of pathogenic bacteria, increases will be found in the volatile end products present in the urine. They may further alter urinary volatiles by

disrupting the balance of normal flora as a result of the competition for substrate (van der Waaij, 1991).

14.2.5. Hormones

The presence of disease may also alter the levels of an individual's corticosteroid hormones. Although steroid hormones are initially transformed in the liver, they are later catabolized by bacterial action (Macfarlane & Cummings, 1991). Thus, the appearance of their metabolites in the urine is actually dependent upon the presence of bacteria. In some instances, an elevation in hormones has also been shown to lead to an increase in the numbers of gut bacteria . Whether this is a result of an increase in the available substrate is not known.

Gonadal steroid hormones change as a result of social interactions among males and reproductive state in females. In these latter cases, discriminable odours result from changes in the levels of these hormones (See Chapter 4). If these discriminations are based upon quantitative changes in metabolites produced by the action of bacteria, then germfree rearing should eliminate these differences also. This hypothesis has not yet been tested.

14.3. The direction of future research

In this thesis, the role of diet cues in producing urine odours of individuality has been established; moreover, the MHC has been hypothesized to interact with diet cues to produce these odours. It also appears that the discriminable volatile end products found in the urine which are controlled by the MHC and diet are expressed through the metabolic activities of gut bacteria. In order to validate the hypotheses, it is imperative to undertake further studies with different strains of rats and mice maintained on different diets and

living in germfree conditions. In collaboration with R. Brown, I hope to undertake such investigations.

APPENDIX I

CLEANING THE OLFACTOMETER

A. CLEANING PROCEDURE

Prior to cleaning the olfactometer, the air supply was turned off; the ductwork was disconnected at the bottom of the main flowmeter ([5] in Figure 6.3; the stimulus jars [15] were replaced with a 13 mm lengths of Teflon tubing and the Teflon tubing leading from the glass manifold [8] was disconnected from the final valve and inserted into a 500 ml glass jar.

Acetone was then flushed through the flowmeter, ductwork and solenoid valves of each stimulus channel in the following manner: The needle valves [4] of all the flowmeters except the one being cleaned were closed. A 50 ml syringe of acetone was injected into the system and the solenoid valves ([13], [16]) of the appropriate channel operated for 25 seconds. The acetone flowed through the ductwork and was evacuated into the glass jar for disposal. The procedure was repeated 3 times so that each channel was flushed with 150 mls of acetone.

To dry the system, the air supply was turned on and the ductwork reattached at the main flowmeter [5]. The needle valves of the main flowmeter and the stimulus channel flowmeters were fully opened. Clean air was passed through the main flowmeter, the stimulus channel flowmeters, solenoid valves and ductwork until all the remaining acetone was evaporated. Each valve was operated thirty times in rotation for 5 seconds each time, until an acetone odour could no longer be detected by a human volunteer sniffer.

B. TESTING FOR CLEANLINESS

To determine if all of the urine residue had been cleaned from the system, three rats which had been trained previously were tested. These animals had proven to be "super-sniffers" in a series of discriminations in which only residual urine was present in the odour delivery system (i.e., no stimulus jars were used).

If these animals did not show any sign of consistent discrimination between any of the stimulus channels (i.e., between air versus air,) in a 200 trial session in which the stimulus jars were removed and the air flow was set at the maximum, the system was considered to be clean. If any of the super-sniffers were able to discriminate between channels in this test, the entire system was cleaned again and these rats given another 200 trials of air versus air discrimination. This procedure was followed until the system was determined to be free from contamination. Prior to any further discrimination procedures, the flowmeters were calibrated to ensure that the air flow was the same in all of the stimulus channels (Appendix II)

C. DISCRIMINATION PERFORMANCE OF SUPER-SNIFFER RATS AFTER THE CLEANING PROCEDURE

Following the cleaning procedure described above, a super-sniffer rat was given the task of discriminating between two stimulus channels from which the stimulus jars had been removed. Figure A1 shows the performance of one animal in five test sessions. This rat was able to discriminate between channels 1 and 4 after three washings with acetone (Figure A1-Session 1). This discrimination of two "clean" stimulus channels could be due to the presence of residual urine or to the presence of acetone in one channel due to an uneven drying process at a concentration not detected by a human sniffer. To test the latter

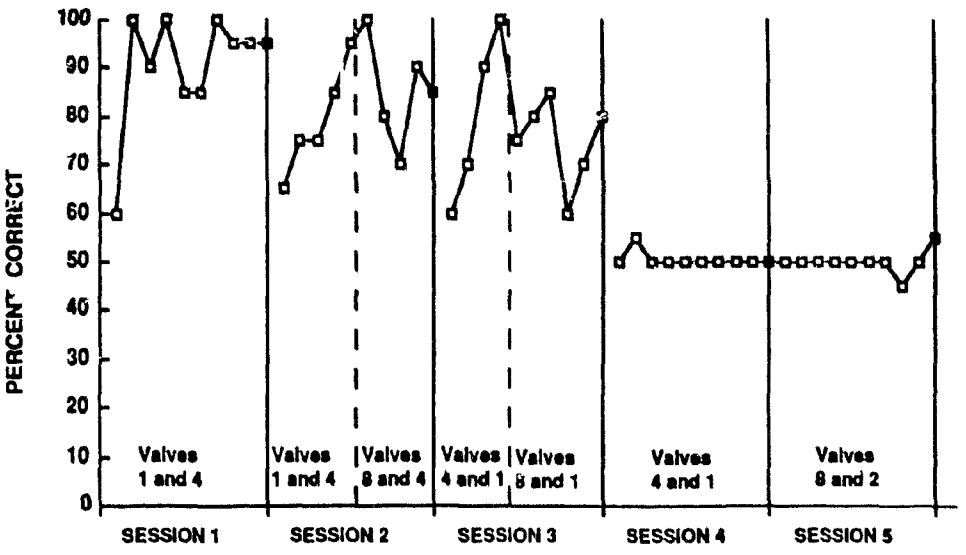


Figure A1: Discrimination performance by a super-sniffer rat when tested on clean air passing through valves which had been cleaned with acetone. The system was cleaned after each session and the valves tested as indicated.

Session 1: Valves 1 and 4 are not clean.
Session 2: Valves 1, 4 and 8 are not clean.
Session 3: All three valves are still contaminated
Session 4: Valves 1 and 4 are clean.
Session 5: Valves 8 and 2 are clean.

possibility each channel was dried by repeating the entire drying cycle (5 sec. by 30 times) for each valve 10 times.

However, the animal's performance in session 2 (100 trials on channels 1 and 4; 100 trials on channels 8 and 4) (Figure A1) indicated that the system was still contaminated, so another 100 mls of acetone was flushed through each stimulus channel to get rid of any residual odours. The "super-sniffer" was still able to discriminate between channels 1 and 4 (Session 3 of Figure A1). As a discrimination was also made between channels 1 & 8, it is evident that the contamination was not limited to channel 4. The entire system was then cleaned with another 50 ml of acetone and dried until acetone could no longer be detected when the valves were operated.

Finally, after five complete clearings, each with 50 ml of acetone, the super-sniffer was no longer able to discriminate between any of the channels (Sessions 4 and 5 in Figures A1) and the system was considered clean enough to begin the next discrimination task.

APPENDIX II

CONSTRUCTION AND CALIBRATION OF THE BUBBLE FLOWMETER

A. CONSTRUCTION

The bubble flowmeter (Figure A2, [1] to [6]) was constructed from a 50 ml graduated glass burette (Kimax) [1] and supported by a ring stand [6]. A rubber bulb [3] was attached to the bottom of the burette via a glass T connector [4]. The stem of the glass T was connected to a tapered glass adaptor [5] via a length of 8 mm Teflon tubing. The tapered end of this adaptor was joined to a 1 M length of 4 mm Tygon tubing [6] which in turn could be attached to the top of a flowmeter ([6] in Figure 6.3).

A 5 ml sample of liquid leak detector (Snoop, NUPRO Co., Willoughby, Ohio) was placed in the rubber bulb to create the soap bubbles necessary to measure the rate of air flow through each flowmeter.

B. CALIBRATION

To ensure that the rate of air flow through each stimulus channel remained constant after each cleaning, each flowmeter was checked and calibrated every time the system was cleaned. In order to measure the air flow, the burette was wetted with liquid Snoop so that the soap bubble would travel up the column and the following procedure was carried out:

First, the needle valves ([4] in Figure 6.3) were adjusted so that the floats in each of the four stimulus channel flowmeters [6] were at the same level. Second, the air flow from the first stimulus channel flowmeter was diverted from the stimulus solenoid valve [13] in Figure 6.3 to the bubble flowmeter through a length of Tygon tubing ([6] in Figure A2); thus, linking the top

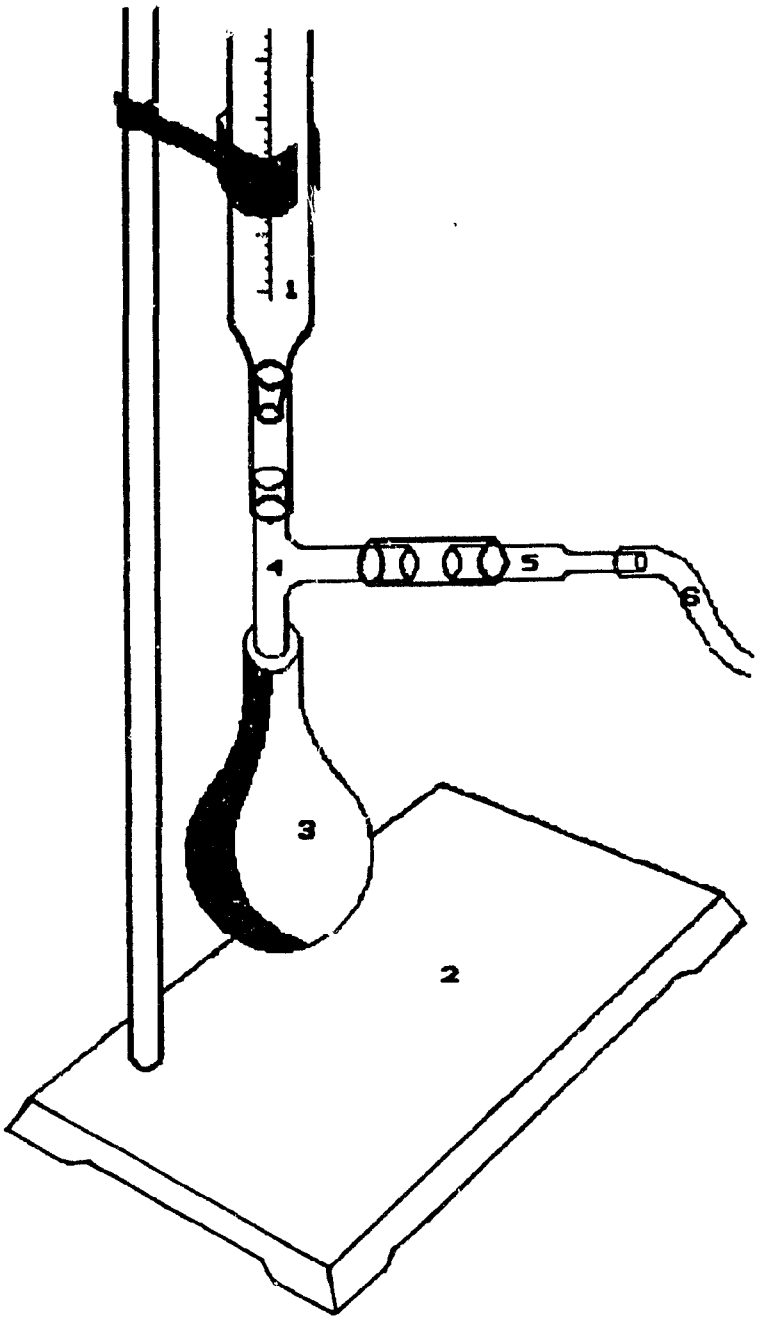


Figure A2: Details of the bubble flowmeter. [1] burette ; [2] ring stand ; [3] rubber bulb; [4] glass T; [5] glass adapter; [6] Tygon tubing.

of the flowmeter to the in port of the bubble flowmeter. A soap bubble was formed by squeezing liquid Snoop out of the rubber bulb [3] attached to the glass burette [1]. The speed (mm/min) that the soap bubble travelled up the wetted glass column of the bubble flowmeter was measured. The value computed was considered equivalent to the flow of air (in cc/min) through the particular flowmeter being calibrated.

This procedure was repeated for each of the remaining three stimulus flowmeters and, if necessary, the air flow was adjusted so that all of the channels were delivering the same amount of air throughout the system. In this study, air flowed through the stimulus jars ([15] in Figure 6.3 at a rate of 120 cc/min. The clean airflow from the compressor pump to the test chamber was maintained at 4000 cc/min.

APPENDIX III

A SAMPLE DATA SHEET

OCT26 89 2<- TIME RAT-->WINCHESTER8
PROGRAM SWEET TWO RAT WINCHESTER8 START LATENCY 79 SEC
S+ WAS GFPVG#8 & #6 DIFFERENT ANIMALS S- WAS GFPVG.R1 #4 & #1

SESSION 56.9 MIN SEC/TRIAL 17 SEC TIME OF DAY 2 OCLOCK
TRIALS COMPLETED 200 ABORTED TRIALS 1 BOX 0
EXPTR HEATHER ITI 3 PUN 0 FI 1.5 RESP AREA 2 FV IS RANDOM TIME
--> --------- RESULTS ---------
%CORR*HITS*COR.REJ*MISS*FALSEALARM*SHORTSAMPLE*MEANITI*CUM-TIME*S+* S-

BLOCK	S+	S-	%	HI	CR	MI	FA	SS	ITI	CUM	RP	RN
1	2	4	55	10	1	0	9	0	9.1	4.6	.47	.52
2	2	4	65	9	4	1	6	1	8.8	9.2	.53	.48
3	2	4	80	9	7	1	3	1	8.7	13.8	.53	.57
4	2	4	85	9	8	1	2	0	11.6	19.5	.57	.5
5	2	4	95	10	9	0	1	0	13.3	25.6	.53	.52
6	1	8	50	7	3	3	7	0	18.9	33.6	.57	.52
7	1	8	50	10	0	0	10	0	15.8	40.4	.62	.63
8	1	8	45	9	0	1	10	0	11.6	45.7	.63	.66
9	1	8	50	10	0	0	10	0	14.0	51.9	.58	.55
10	1	8	55	10	1	0	9	0	10.4	56.9	.56	.52

TOTAL ERRORS 74 MEAN SAMPLING TIME FOR S+ IS .55 FOR S- IS .54

MEAN SAMPLE TIMES/# TRIALS OF EACH TYPE

BLOCK	HITS	CR	MI	FA
1	.46/10	.66/1	.00/0	.50/9
2	.52/9	.46/4	.57/1	.49/6
3	.52/9	.58/7	.53/1	.51/3
4	.57/9	.53/8	.53/1	.39/2
5	.52/10	.51/9	.00/0	.58/1
6	.55/7	.52/	.60/3	.52/7
7	.62/10	.00/0	.00/0	.62/10
8	.60/9	.00/0	.83/1	.65/10
9	.57/10	.00/0	.00/0	.54/10
10	.56/10	.71/1	.00/0	.49/9

ZERO TRIALS PRIOR TO FIRST REINF TRIAL
TOTAL REINF 95 TOTAL WATER OBTAINED 1.9 ML

INITIAL COMMENT NOT A GREAT DISCRIMINATOR

FINAL COMMENT A PERFECT EXAMPLE OF NON-MHC DISCRIMINATION

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