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MATERNAL PHEROMONE IN THE RAT

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by

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Summary of Thesis submitted for Ph.D. degree

by F.M. Clegg,

on

Maternal Pheromone in the Rat

An integrated series of experiments was conducted to investigate an initial failure to replicate the maternal pheromone effect in the rat. It examined:

1. Apparatus parameters (airflow rate)

2. Pup factors (age, pre-test maternal deprivation, early experience)

3. Maternal factors (diet, strains; PVG/C, Wistar, Sprague-Dawley)

Publications on the maternal pheromone present a miriad of problems; methodological, conceptual and statistical, and a visit to two of the North American laboratories, where work on the pheromone is being pursued, failed to resolve these difficulties. The phenomenon is not as robust as the literature would suggest.

Final experiments, using a simplified method, showed that pre-weanling rats are influenced by olfactory cues; throughout the whole study the young had shown a slight preference for maternally derived odours.

Use of the term "pheromone", in connection with mammalian behaviour, is criticised. In particular it is concluded that there is insufficient evidence to support the existence of a maternal pheromone in the rat.

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I remember the rage I used to feel when a prediction went awry. I could have shouted at the subjects of my experiments "Behave, damn you. Behave as you ought." Eventually I realised that the subjects were always right. It was I who was wrong. I had made a bad prediction.

B.F. Skinner.

CHAPTER I

PHEROMONES

Origin and Meaning of the term "Pheromone"

The term "pheromone" was coined in 1959 by the entomologists Karlson and Lüscher. The word is made up of the two Greek words "pherein" - to transfer, and "hormon" - to excite, and it refers to active chemical substances which are produced by living organisms and released into the external environment. Here, they influence the behaviour of other members of the same species.

It had been known for a long time that most living organisms produce secretions which act internally (hormones); the newly discovered external secretions were at first thought to be rather similar, and until 1959 they were called "external hormones" or "ectohormones" (Bethe, 1932) until 1959. Whilst hormones affect specific receptor sites within the organism, the externally secreted substances have to be detected by peripheral receptors in the perceiving organism. These receptors comprise the systems known as the "chemical senses", and the closely interlinked senses of taste and smell are the two known to be present in man.

It is possible for living organisms to detect and respond to substances secreted by individuals of the same or different species, but the term "pheromone" does not have such a broad application. The original definition put forward by Karlson and Lüscher is: "....substances which are secreted to the outside by an individual of the same species in which they release a specific reaction, for example a definite behaviour or developmental process." The importance of substances serving as messengers within a particular species is brought out in this definition, and also, the fact that pheromones are seen to release a specific reaction, somewhat similar to the ethologists' Fixed Action Pattern, in the recipient.

During the 1960's, there was an increased awareness on the part of research workers of the importance of chemical communication systems, both within and between species, and attempts to formalise and classify them were made. Law and Regnier (1971) named all the chemical substances which served in a communication capacity "semiochemicals", and divided them into two classes; chemicals which serve as messengers between members of the same species (intraspecific), and those with the same function, but which act between species (interspecific). These writers divide the interspecific semiochemicals according to whether their transmission adaptively favours the producer or the receiver, and these are called "allomones" and "kairomones", respectively. They give the name "pheromone" only to the intraspecific messenger substances, and these are not subdivided in their scheme.

Other classifications differ slightly. Whittaker and Feeny (1971) call all the interspecific chemicals "allelochemics", and form three subdivisions; "allomones", "kairomones" and "depressants", the division again being according to adaptive advantage for receiver or sender. They also divide the intraspecific messenger substances in the same way, and name the three categories "autotoxins", "adaptive autoinhibitors" and "pheromones".

Whilst there is general agreement that pheromones constitute a type of intraspecific messenger substance, several researchers have suggested that they should be subdivided, but they offer different criteria. Karlson (1960) separated olfactory and gustatory pheromones; Butler (1967) classified them on the basis of their biological function; Shorey (1973) on the basis of the kinds of behaviour which they evoke, and Wilson (1963) distinguished pheromones which bring about an immediate effect - "releasers", from those which evoke a long term and less specific reaction, the "primers". Wilson's division seems to be a popular one, and has been successfully used in connection with insect behaviour, where sophisticated means of chemical analysis have enabled entomologists to obtain and identify very precisely some mediating substances.

Perhaps the most impressive feature of this research is the minute quantities and tremendous potency of the substances involved. The quantities of insect attractants which may bring about responses in the appropriate recipient can be counted in molecules, whilst the sensitivity of the chemical receptor systems has been called "unbelievable" (Wilson, 1963). Naturally it is hard for creatures who rely mainly upon their visual apparatus to give credence to a world interpreted through chemical senses; perhaps this is one of the reasons why systematic research into, and control of olfactory variables has until recently been almost completely neglected in mammalian research.

By 1970 it had been established that pheromones were involved in several types of vertebrate and invertebrate intraspecific interactions, and Whittaker and Feeny (1971) list six main kinds:

1. Reproductive behaviour
2. Social regulation and recognition
3. Control of caste differentiation
4. Alarm and defence
5. Territory and trail marking
6. Food location

Under the heading of reproductive behaviour comes "maternal behaviour". This refers to the extremely effective pattern of behaviour shown normally by the female, but occasionally by the male parent in caring for the vulnerable offspring. Although much of this behaviour, of an apparently unlearned and relatively stereotyped nature appears to be under hormonal control (Lamb,1975; Lehrman, 1961; Rosenblatt, 1969), it would appear that exteroceptive factors play a role in such behaviour, and particularly in its maintenance (Grosvenor and Mena, 1973; Deis, 1968; Terkel and Rosenblatt, 1971). It is not unreasonable to suppose that certain responses may be triggered off by pheromones, one type of exteroceptive stimulus, as has been demonstrated in many other aspects of reproductive behaviour.

A brief description of some of the most interesting and important of the discoveries made in this area, and the putative role of pheromones in maternal behaviour will follow in the next sections.

Psychological investigations into the sense of smell

Humans have for many centuries expressed the view that animals enjoy a much keener sense of smell than they do themselves. At the end of the nineteenth century smell was mentioned by several biologists and early psychologists, and in his study "Notes on the Psychic Development of the Young White Rat", Small (1899) comments on the reactions of baby rats to various substances which he places near them. He concludes from the varied reactions which he observed that rats move from a relatively undistinguishing sense of smell at birth towards a finer sensitivity by puberty, and also that considerable individual variation exists between rats from a very early age.

Whilst psychologists like King, working at the Wistar Institute with the first laboratory rats, carried out observational studies (King, 1939; Donaldson, 1924; Wiesner and Sheard, 1933), many psychologists started to address themselves to problems such as whether behaviour patterns like "mating", "maternal care" or "nest-building" were instinctive or learned. Another stream of research was directed towards such things as learning ability, memory processes and emotion in the rat, with the hope of discovering and understanding underlying principles which could then be applied to human behaviour. Both types of research have been criticised; the former for being too simple and the latter for inappropriate comparison.

Perhaps another criticism would be to accuse psychologists of that period of anthropomorphism, in that they based their experimental work on the assumption that inside every rat skull was an inferior version of the human brain - a brain in which the most important sensory modality was vision. The chemical senses were ignored by most experimental psychologists; maybe they were following William James's lead. In his "Text-book of Psychology", first published in 1892, James devotes chapters to sensation in general, sight, hearing, touch, the temperature sense, the muscular sense and pain, motion, the sense of time, perception and the perception of space. Taste and smell are not mentioned. The chemical senses are so unimportant in human behaviour (or appear to be unimportant), that their omission from the literature did not seem to be of much consequence. A serious result, however, was that the neglect was not confined to the study of human behaviour, but also occurred throughout almost fifty years of animal experimentation. Conclusions were drawn from research which failed to consider, let alone control, variables relating to the chemical senses.

In 1873 Spalding observed and wrote about the phenomenon which was later named "imprinting" by Lorenz (1935). In his paper, which was to pave the way for much of the research done on the nature-nurture debate and the concept of the "critical period", Spalding notes what he calls the "....unacquired power of following by sight." During subsequent years, in research carried out mainly on birds, sight remained the modality which was the focus of attention. It was not until 1965 that Mainardi et al. suggested that a parallel phenomenon might occur in young mammals, but based on odour (i.e. chemical stimulation) rather than vision. Whilst people engaged in such occupations as farming have always been aware of the role of smell in successful mother-young relationships, it is only relatively recently that scientists have started to undertake systematic research on farm animals. Work on the role of odour in reproductive behaviour in pigs has been reported by Booth (1975), Signoret (1970) and Melrose et al. (1971); in sheep by Lindsay (1965) and Banks et al. (1963); in cattle by Donovan (1967), and in goats by Klopfer and Gamble (1966).

By the late 1960s, psychologists working on the so-called "instinctive" behaviours had managed to correlate many patterns with underlying hormonal events, and the possibility that the chemical senses had a role to play in these, in addition to hormones, began to be considered by many investigators. Papers in which the sensory parameters of instinctive behaviour were delineated appeared, and the variables associated with chemical sense perception were sometimes controlled (Myer, 1964; Noirot, 1970; Beach and Jaynes, 1956), or even studied in their own right (Grosvenor and Mena, 1973; Fleming and Rosenblatt, 1974; Gandelman et al. 1972).

The tremendous success enjoyed by entomologists at this time, in their explanations of many of the ways in which insect behaviour is apparently controlled by chemical mediation, made it seem almost inevitable that psychologists should take a leaf out of their book, and pursue a similar line of enquiry. Speculation as to the role of the chemical senses in many aspects of mammalian behaviour increased, and after about half a century of oblivion, the entry of this modality into the psychological arena was full of promise.

By 1970 olfaction had replaced vision as the modality considered to be of most importance in mediating the behaviour of many mammals. In particular, reproductive behaviour in rodents had undergone detailed scrutiny, and several interesting findings had been made. The most important phenomena were named after their discoverers: the Bruce, Whitten, Lee-Boot and Vandenberg effects.

The Bruce effect (Bruce, 1959) refers to the finding that when a female mouse is taken from her mate and exposed to the odour of a strange male within a day of impregnation, then the pregnancy is blocked and she returns to the oestrous phase of the reproductive cycle within a week.

Synchronisation of cycling which occurs in the female mouse after exposure to the odour of a male mouse became known as the Whitten effect (Whitten, 1956); it was discovered after further investigation of the Lee-Boot effect - the synchrony of oestrous cycling which female mice will start to display when they live without the odour of male mice in their environment (Whitten, 1959; Lee and Boot, 1956).

If female mice are reared from birth in an environment which is free of male mouse odour, then they are slower in attaining their first oestrous than if they live in a mixed sex environment. The stimulating effect which some component of the urine from a male mouse has on the growth rate of pre-pubertal female mice is called the Vandenbergh effect (Vandenbergh, 1969).

A detailed and excellent summary of many of the discoveries made on the role of pheromones in reproductive behaviour in rodents is provided by Bronson in a review of pheromones in both vertebrate and invertebrate species edited by Birch (1974).

Interesting work on the role of the chemical senses in investigatory behaviour in rodents has appeared recently. Nowell and his colleagues have demonstrated that one of the components of mouse urine, an androgen-dependent substance, influences investigatory behaviour by male conspecifics (Jones and Nowell, 1973b, 1974), and a result of this work has been the discovery of an aggression-inhibiting substance, which is probably the same chemical (Jones and Nowell, 1973a, b; 1975).

Valenta and Rigby (1968) showed that a rat experiencing noxious stimulation could leave olfactory cues which would influence the behaviour of a later visitor of the same species to the spot. Morrison and Ludvigson (1970) have also published evidence of a similar nature, using rats which were run in a T-maze for a reward of food, or no reward.

These findings cast doubt upon the validity of much of the early work done by learning theorists using rats in single pieces of apparatus. It seems likely that in many instances the rats were not responding to the varying values of the independent variables, but to uncontrolled, often confounding, variables provided by chemical secretions whose presence was undetected and unimagined by the experimenters. As researchers become increasingly aware of the important role of chemical messenger substances and the chemical senses in mediating animal behaviour, it is to be hoped that this is reflected in more adequate experimental methodology.

Investigations undertaken by psychologists into the role of the chemical senses in behaviour have usually been by means of behavioural bioassay. An assay is a test in which the object of examination, by manipulation of the independent variable, is subject to a number of changes. Its reactions are recorded, thus yielding quantitative data for analysis. Bioassays involve the study of living animals or their tissue and behavioural bioassays are tests in which responses of the whole organism, i.e. its behaviour, are quantified.

On the whole, in psychological studies involving the chemical senses, it has not been possible to name specific chemicals as agents in particular behavioural responses. The behavioural bioassays are relatively crude - for instance merely measuring approach or avoidance behaviour shown after exposure of the organism to various organic compounds. There are some exceptions however; one lies in the study of reproductive behaviour in pigs, where much is known about the steroid hormones involved (Signoret and du Mesnil du Buisson, 1961; Signoret, 1964; Melrose et al., 1971; Reed, Melrose and Patterson, 1974).

In simpler animals, where control can be more rigorous and the behaviour patterns are less complex, detailed knowledge of chemical substrates which influence behavioural responses exist, and entomologists have enjoyed tremendous success in their accounts of chemically guided behaviour in insects. Often, after having demonstrated that a particular chemical was involved in the control of behaviour, they would then refer to the substance as a pheromone.

It is of interest to compare the use of the word "pheromone" with the term "instinct", as there are certain similarities between the way that the use of them has altered. Despite the fact that "pheromone" originally had a precise definition, psychologists began to apply it in a rather loose fashion to almost any olfactory cue which elicited some kind of behavioural response. Subsequently, "pheromones" have been used to provide explanations for any behaviour apparently mediated by the chemical senses. The term "instinct" was originally used to describe certain types of relatively stereotyped, unlearned behaviour; gradually it acquired an explanatory connotation, and soon became a term with little use. As the former "instincts" were gradually explained more adequately in terms of underlying physiological variables, the old umbrella label ceased to be of value. The same thing is likely to happen to the term "pheromone". At the moment the term is being used increasingly in an explanatory capacity. Whilst "pheromone", applied indiscriminately to olfactory cues can be regarded as merely misleading, the term offered as an explanation for kinds of behaviour which are complex and variable - not highly specific, as in the original definition, is an error. It might be that the trend to use the word in this manner will halt when the chemical substrates of certain behavioural responses are known, and like "instinct", "pheromone" will no longer serve a useful purpose.

Meanwhile, there has been speculation as to whether pheromones might mediate certain types of human behaviour, and this has caught the imagination of some popular writers. Further discussion of the term "pheromone", and whether pheromones might play a part in primate behaviour will be considered in Chapter XVI.

<u>The Maternal Pheromone</u>

The role of olfaction in reproductive behaviour has been studied quite extensively in mice, but even by 1970, little work had been carried out on rats. The most noteworthy publications were those by Beach and Jaynes (1956), Carr <u>et al.</u> (1961, 1965), and Le Magnen (1951, 1952).

Maternal behaviour in rats was by this time well documented (Munn, 1950; Lehrman, 1961; Rosenblatt and Lehrman, 1963; Richards, 1967; Rosenblatt, 1970). A short time before parturition a pregnant female rat starts to exhibit increased nest-building behaviour, and on giving birth to her young, she will remove the fine membrane enclosing each pup, lick the pup clean and eat the placenta. Constant licking by the mother of the pups' ano-genital area ensures that they develop the ability to empty their bladders. Young are kept in a single location in the nest, often covered by the mother, who shows a big decrease in activity and adopts a crouching position, enabling the young to feed for several hours a day. In the mother's absence, the pups are covered over with nesting material. When the pups become mobile and leave the nest, the maternal female rat will carry them back to it, and this behaviour, known as "retrieval" is exhibited straight after parturition if the young are scattered about the home cage, or if new young are placed in it. Retrieval is shown by the lactating female rat until about 14 days post-partum (Rosenblatt, 1965), when its frequency declines sharply. Although after this age the pups continue to spend a large proportion of their time feeding, this behaviour is initiated by them, in that they actively approach the mother, rather than by the mother (Rosenblatt, 1969).

Indices of maternal behaviour, usually quantification of nest-building activity, attention to pups and retrieval have been developed, and are commonly used in studying mother-young interaction. It has commonly been supposed that the female rat is the main agent in the survival of the young, through her display of these activities. More recently, attention has been turned to the young themselves, and their role in ensuring their own survival. It seems that they emit cues which appear to trigger off maternal responses in the mother, and they are also capable of certain activities immediately after birth, e.g., moving towards a warm object, locating a nipple on the mother's ventral surface and then through suckling, keeping themselves firmly attached to the lactating female.

The neglect, until recently, of the chemical senses in the study of maternal behaviour has been equalled only by that of auditory cues. Although it was known in 1954 that laboratory rats emit ultrasounds (Anderson, 1954), there were very few reports on this topic until the mid-1960s. Noirot, working with mice, demonstrated that ultrasound facilitates maternal responses by both inhibiting aggression and stimulating nest-building and retrieval (Noirot, 1964, 1968, 1970). It is now known that rat pups emit ultrasonic distress calls when they are handled (Sales, 1972) or when their temperature falls (Okon, 1970), and that such calling can bring about orientation and retrieval by the mother (Allin and Banks, 1972). Indeed, rodents will respond to auditory cues from other species, and artificially generated sounds (Smith, 1975). As yet, there has been no publication in which a synthesis of the many different exteroceptive and interoceptive factors known to be involved in maternal behaviour has been attempted.

It had always been thought that the mother was the main agent in ensuring that the young stayed in the nest, but in 1970, the Hungarian researchers Nyakas and Endrőczi looked at the constant uniting of mother and young in an unusual way. They considered the notion that the young themselves move towards the mother, being attracted either to her body, or to her secretions. They found that after 5 hours of maternal deprivation, 10 day old rat pups would select the arm of a maze which the mother had previously occupied. Maternal urine would elicit orienting behaviour, and local application of tetracaine to the pups' nares, which blocked the sense of smell, eliminated approach behaviour to a certain extent (Nyakas and Endröczi, 1970). The idea that olfactory cues play an important role in keeping the young in the nest was quickly taken up by Leon and Moltz (1971) who, after modifying the original apparatus and procedure, also found that young rat pups moved towards a source of maternal odour. They labelled the olfactory cue believed to be involved the "maternal pheromone" - this was the first time that the term appeared in the literature, and the first time that psychologists chose to use the label "pheromone" in connection with mammalian behaviour.

> "It now seems likely that the cue uniting mobile pups with their lactating mother is olfactory in nature which demonstrates that a pheromone functions to synchronize the mother-young relationship in the laboratory rat."
>
> Leon and Moltz, 1971.

Although many animal behaviourists continued to refer to "olfactory cues" in their work on olfaction in mother-young interaction in other species, rather than adopt the new term (Gregory and Bishop, 1975; Porter and Etscorn, 1974; Devor and Schneider, 1974), the label was used in subsequent work by Leon and Moltz and their colleagues, and later taken up by textbook writers and reviewers. The maternal pheromone refers to a chemical substance, probably secreted in the anal excrement of lactating rats, which, when detected by pre-weanling rat pups, causes them to approach the source of the odour.

The next chapter comprises a review of the experimental investigations carried out into the maternal pheromone.

CHAPTER II

THE LITERATURE ON THE MATERNAL PHEROMONE, 1971 - 1975

INTRODUCTION

Following the finding by Nyakas and Endroczi (1970), that 10 day old rat pups were attracted to the mother or her secretions, Leon and Moltz, working at Chicago University, carried out a modified version of the experiment, confirmed the effect, and named the attractant which they believed to be involved, "maternal pheromone". They then went on to carry out a series of experiments in which they investigated the maternal pheromone further. Subsequent papers published by Moltz, either with Leon or other colleagues, delineate the parameters of maternal pheromone production and emission, whilst the two papers written by Leon alone (1974, 1975), develop a complex model of pheromone production.

In 1970 Schapiro and Salas demonstrated that 2 - 12 day old rat pups' activity levels are influenced by an odour derived from the mother. Soon after this, and at about the same time that Leon and Moltz's first publication on the maternal pheromone appeared, Gregory and Pfaff (1971) also published findings on olfactory guided behaviour in infant rats. These papers are important, as they provide additional evidence that olfactory cues have an effect on the behaviour of pre-weanling rat pups. They are also typical of many later papers on the same topic, in that they refer to "olfactory cues" or "odour preferences", rather than "pheromones".

Very few of these later investigators worked with rats however, and none of them used apparatus resembling the olfactory discrimination apparatus built by Leon and Moltz.

Because Leon and Moltz and their colleagues use the olfactory discrimination apparatus in all their work, and as it is of central importance to the research findings described in this chapter, it is described in detail in Part I, along with the accompanying test procedure.

PART I

The Olfactory Discrimination Apparatus

The olfactory discrimination apparatus comprises a triangular "open-field" made out of plexiglass, with two 23 x 23 x 28 cm goal boxes lying along the base of the arena, which faces a 10 x 10 x 13 cm start box on the apex of the triangle. The goal box doors are made of opaque plexiglass, so that the goal box contents are not visible from the arena, and they extend downwards to a small ledge 5 cm below the level of the arena floor. A current of air is forced into each goal compartment from a central valve, entering through 70 mm holes located below the grid floors of the goal boxes, passing from there up over the ledge into the apparatus arena, and from there to the start box.

The olfactory discrimination apparatus is shown in Figure 1, which is the diagram given by Moltz (Moltz, 1975). Some details necessary for the construction of an identical piece of apparatus are missing. It is not known whether the walls of the arena are made from opaque or clear plexiglass. The way in which the start box and goal box doors open to allow the pups through is not indicated, although as the goal box doors are shown as being right up to the arena, it was assumed that they are hinged at the top, and open by swinging inwards. The width of the ledge under the goal boxes is not given, neither its distance below the arena floor, although this is stated to be 5.08 cm in the monograph "Maternal Pheromone" (Leon, 1974). In this publication, the reader is also informed that filtered air is passed through the apparatus at a rate of 55 litres/min, and that the airflow pump created a constant masking noise of 72 dB A Scale. Polythene-backed absorbent paper covers the open-field floor.

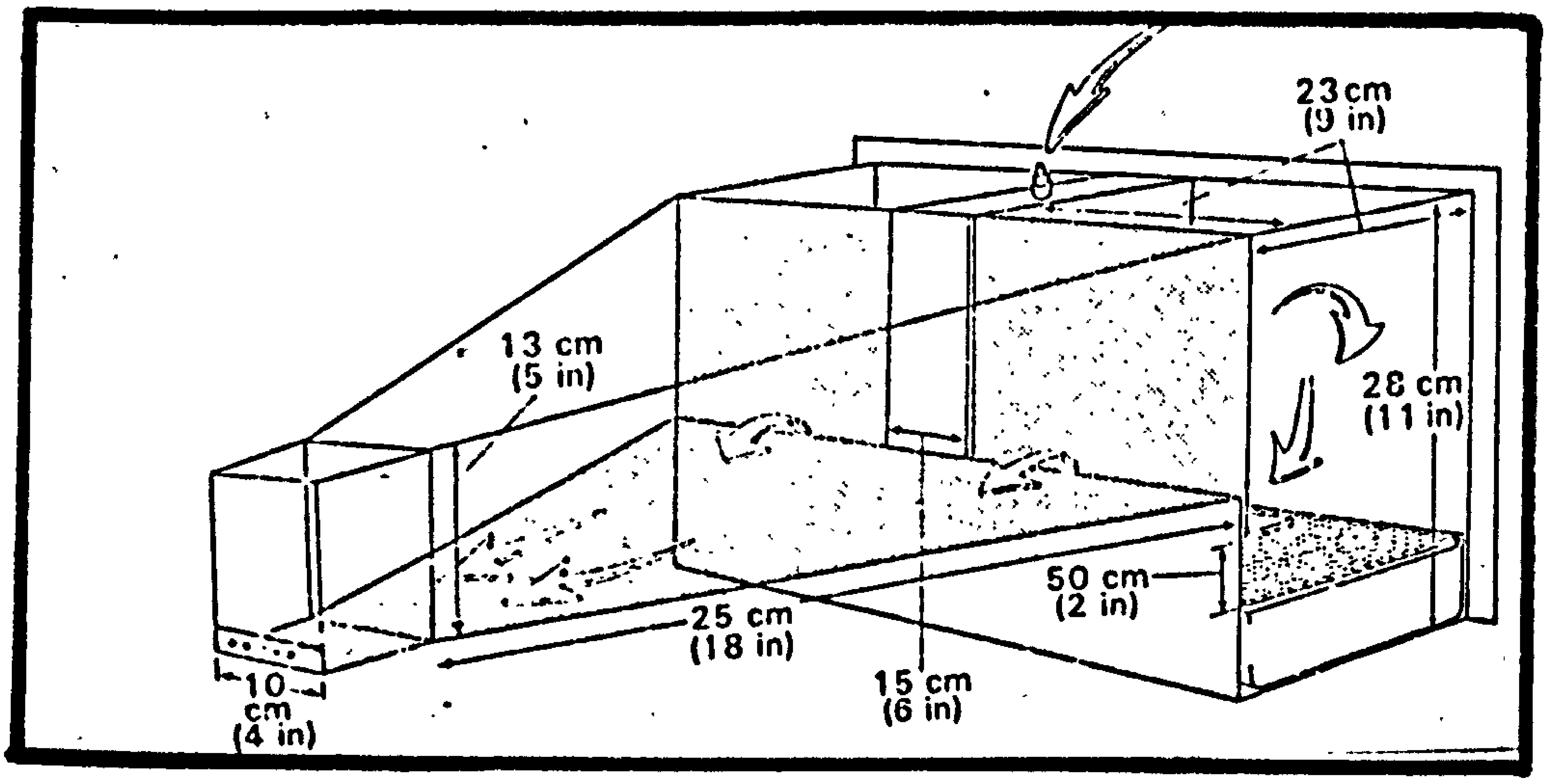

Figure 1. The olfactory discrimination apparatus. Arrows indicate the direction of airflow.

(From Moltz, 1975)

The Standard Test Procedure

Rat pups were deprived of maternal attention by removing the mother from the home cage for 3 hours before the start of a testing session. When testing started, the stimulus material (which varied according to the particular experiment, but which was usually an adult animal, or some kind of excrement) was placed in the goal boxes on the wire grid floor, and the rat pup then placed in the start box before being allowed to enter the arena.

The choice behaviour of the pup was then recorded. For pups older than 12 days of age, descent into one of the goal boxes was registered as a positive choice. Otherwise, and after 15 minutes had elapsed, the behaviour was recorded as "no choice". For pups younger than 12 days the criteria constituting a choice were less stringent. Day 1 pups were required to crawl at least 20 cm from the start box, and the side they moved along was recorded as their preference. Day 10 and 12 pups had to approach a goal box ledge for their behaviour to be recorded as a positive choice.

After each pup had been tested the absorbent paper cover in the arena was changed, and after 3 pups had been run, the whole apparatus was washed out, and the goal box contents exchanged. All pups were given only one trial in the olfactory discrimination apparatus.

PART II

Leon and Moltz's Findings

The publications by Leon and Moltz and their associates are given in chronological order in Table 1, and this section presents a brief outline of the main results reported in each of these papers. The emergence of new concepts or lines of research is also noted.

As Leon and Moltz worked in separate laboratories after 1973, all Moltz's work from the original laboratory in Chicago will be followed first, with a return to Leon's concurrent work at McMaster University in Canada.

Table 1

Publications by Leon, Moltz and their colleagues; 1971 - 1975 listed in chronological order

YEAR OF PUBLICATION	TITLE	AUTHOR AND LABORATORY
1971	Maternal Pheromone: Discrimination by Pre-Weanling Albino Rats	Leon & Moltz (Chicago)
1972	Development of the Pheromonal Bond in the Albino Rat.	Leon & Moltz (Chicago)
1973	Stimulus Control of the Maternal Pheromone in the Lactating Rat	Moltz & Leon (Chicago)
1973	Endocrine Control of the Maternal Pheromone in the Postpartum Female Rat	Leon & Moltz (Chicago)
1974	Prolongation of Pheromonal Emission in the Maternal Rat	Moltz, Leidahl & Rowland (Chicago)
1974	Maternal Pheromone	Leon (McMaster)
1975	Dietary Control of Maternal Pheromone in the Lactating Rat.	Leon (McMaster)
1975	Emission of the Maternal Pheromone in the Nulliparous Female and Failure of Emission in the Adult Male	Leidahl & Moltz (Chicago)

All the publications listed in Table 1 appear in the Journal <u>Physiology and Behavior</u>.

(i) Maternal Pheromone: Discrimination by Pre-Weanling Albino Rats

Leon and Moltz, 1971

Rat pups, aged 16 days, were deprived of maternal attention for 3 hours and then given a choice in the olfactory discrimination apparatus between either their own 16 day lactating mother; a strange 16 day lactating mother; a nulliparous female and an empty goal box - in various combinations. In one set of trials the two goal boxes were soiled by a strange mother and a nulliparous female before testing started; in another set, where the choice was between the mother and a nulliparous female, the direction of airflow was reversed. Apart from the trial in which the mother was opposed to a nulliparous female, when pups were run singly, all pups were tested in groups of three.

The results suggested that 16 day lactating females emit an odour which attracts 16 day old rat pups, and that it is not litter specific. The odour, described as discriminable by humans, forms the basis of the cue which Leon and Moltz call a pheromone, and whose role, they suggest, serves "....to synchronize the mother-young relationship in the laboratory rat.".

(ii) The Development of the Pheromonal Bond in the Albino Rat

Leon and Moltz, 1972

The first of the papers in which the parameters of the maternal pheromone are delineated, the purpose of this investigation was twofold; to determine at which stage of lactation and for how many days the maternal pheromone is emitted, and to discover over which age-range the young respond to the pheromone. Pups were all tested singly, and the choice criteria for pups younger than 16 days are the ones which were given in Part I of this chapter.

Four experiments are reported. First it was shown that the young begin to approach their own mother in preference to a nulliparous female at 14 days age. The mother was overwhelmingly chosen until Day 21 post-partum, after which the attraction waned, until it had disappeared by Day 41. As the age of the young was confounded with the mothers' stage of lactation, a refinement of this experiment was run, in which young aged from 1 - 41 days were given the choice of a 16 day lactating female opposed to a nulliparous female. Similar results were obtained, with the peak of the pups' responsiveness occurring between 12 and 27 days age. Next, at 16 days (the age of maximum responsiveness) young were given the choice of mothers at different lactational stages, opposed, in Experiment 3, to a non-lactating female, and in Experiment 4 to a 16 day lactating female. Leon and Moltz conclude from their findings that lactating females emit the pheromone in an all-or-none fashion between 14 and 24 days post-partum.

This paper is the first one in which the term "bond" is used. The experiments "....revealed the existence of a striking synchrony in the development and subsequent dissolution of the pheromonal bond in the albino rat.". The writers then say, "....it is of interest to speculate about the role such a bond might play in synchronizing the mother-young relationship.". They suggest that it serves to unite mother and young at the time when maternal retrieving declines, and continues until the weaning process starts. At this stage (about Day 27) not only does the mother cease to emit the pheromone, but the young also cease to be attracted to it.

(iii) Endocrine Control of the Maternal Pheromone in the Postpartum Female Rat

Leon and Moltz, 1973

Physiological mechanisms underlying the 14 day period of pheromone emission are investigated in this series of experiments, and the hormonal agents believed to be involved are examined. Lactating female rats were subject to a variety of operations - adrenalectomy, ovariectomy, or both operations, so that hormone release was disrupted. Additionally, a group of mothers was given ergocornine hydrogen maleate at a dosage known to interfere with prolactin release. As some mothers would experience lactational failure after surgery, all females were given daily litter replacements of young of advancing age. Testing at 16 days post-partum, was with "standard" 16 day colony young.

Adrenalectomy, ovariectomy and the combined operation did not interfere with pheromone emission. However, prolactin inhibition did supress pheromone release, and in the control group given exogenous prolactin along with ergocornine, emission on Day 16 was normal. The writers suggest that work needs to be carried out on totally mammectomised females to resolve conclusively the question of lactational involvement - the problem being that in just one group which did not maintain lactation, the pheromone was produced normally. Despite some problems of interpretation, the following unambiguous statement appears in the Discussion section:

> "The present studies demonstrate conclusively that the maternal pheromone is under prolactin control."

(iv) Stimulus Control of the Maternal Pheromone in the Lactating Rat

Moltz and Leon, 1973

The possibility that the stimulus characteristics of the offspring govern the emission of the pheromone is explored in this paper.

With one group of females, litters were removed at parturition and replaced daily for a period of 16 days by 1 day old pups. A second group of newly parturient mothers were given foster litters 10 days old, and tested 6 days later. Suitable control groups were run, and test animals were 16 day young from the colony. It was found that when a mother was kept continuously with the day old young she did not emit the pheromone; neither was its appearance advanced by the substitution of older pups. The influence of stimulus characteristics on pheromone emission is labelled "asymmetric", because it seems possible only to delay emission, not to advance it. Results are discussed with reference to the finding already outlined in "Endocrine Control of the Maternal Pheromone in the Postpartum Female Rat", namely that prolactin production is involved in pheromone emission. The writers point out that in attempting to advance the onset of pheromone production, 6 days may not have been an adequate period for high prolactin levels to develop and give rise to its emission, but suggest that injections of prolactin should remedy this deficiency.

(v) Prolongation of Pheromonal Emission in the Maternal Rat

Moltz, Leidahl and Rowland, 1974

In this report, Moltz and his colleagues continue to delineate the parameters of pheromone emission, but now working without Leon, who moved to McMaster University, Hamilton, Canada.

The results of Experiment 1 indicated that given the continuous stimulation from pups aged 14 - 21 days, mothers will emit the pheromone well past the normal 27 day limit, and for more than 100 days post-partum in some cases. If, however, young of advancing age were kept with a pheromone producing mother until 60 days post-partum (Experiment 2), they cease to respond to the attractant at the usual age of 21 days. Asymmetry is again described, this time being that whilst the mothers' pheromone release is sensitive to the character of the young, the young are apparently insensitive to the state of the mother.

(vi) Emission of the Maternal Pheromone in the Nulliparous Female and Failure of Emission in the Adult Male

Leidahl and Moltz, 1975

If nulliparous female or male rats are given new-born rat pups daily for several successive days, a large proportion of them will start to behave in a maternal fashion; nest-building, retrieving and assuming a crouching posture over the pups, as if nursing. This technique for artificially inducing maternal behaviour through constant exposure to pups, is known as "concaveation". It was used by Leidahl and Moltz in the experiments reported in this publication.

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After groups of female and male rats which had been concaveated first showed maternal behaviour (and not <u>all</u> the rats were successfully concaveated), the daily substitution procedure was continued, but the day old pups were now replaced by pups of gradually advancing age. When these pups were 16 days old (i.e. 16 days had elapsed since the first appearance of maternal behaviour), the concaveated animals were tested in the usual way, matched against a nulliparous female which had not been continuously housed with young, and with 16 day colony young making the choice. It was found that whilst concaveated females emitted the pheromone, and for a longer period than normal mothers, the males did not emit any attractant at all, even though they displayed impeccable maternal behaviour. A group of females which had been housed with day old young for many days, but which had failed to display maternal behaviour, had been yoked to the successfully concaveated females, but they also failed to emit the maternal pheromone.

The conclusion drawn by the authors is that a display of maternal behaviour in the female is an essential precondition for pheromone emission, but that in the male, for some reason, although maternal behaviour <u>is</u> displayed, emission is not possible. Some discussion of the failure of the male to emit the pheromone follows; the investigators contradict a conclusion which they stated had emerged "clearly" from the experiment, in that they advance the notion that males <u>are</u> emitting the pheromone, but that its attractive properties are masked by the presence of an androgen metabolite "....perhaps present in the feces". However, the reader is assured that questions arising from this investigation are giving rise to further experimentation, in the hope that definitive answers will be secured.

(vii) Maternal Pheromone

Leon, 1974

After a brief summary of the earlier finding that young rat pups are apparently attracted to an olfactory cue emanating from a lactating female, Leon reports a series of experiments designed to investigate the underlying control of the pheromone synthesis and emission. The work carried out by Leon, in collaboration with Moltz between 1971 and 1974 is not summarised in a formal manner, although passing reference is made to several of the earlier experiments throughout the monograph. Until this time, all earlier conclusions have been based on work in which live, mobile animals were placed as stimulus material in the goal compartments (with the exception of one set of trials in the very first report), but now Leon starts reporting work in which excremental material rather than animals is used almost exclusively.

Firstly, he demonstrates that young are attracted to maternal anal excrement (but not urine), over the same 7 day period of lactation as they had been attracted towards the mother in the earlier experiment (Leon and Moltz, 1972); secondly, that the source of the odour is caecotrophe, an unformed type of anal excrement which is normally eaten by rats, but which is produced in large quantities by lactating females; thirdly, that the pheromone is synthesised in the caecum rather than produced in the anal glands; and fourthly, that a similar, if not identical, attractant is to be found in the caecal contents of virgin females. When lactating females were given an antibiotic which inhibited bacterial growth in the caecum, or a diet without raw carbohydrate (which deprived the bacteria of a growth medium), the caecotrophe lost its pheromonal properties. Leon goes on to suggest that prolactin is involved in the production of the maternal pheromone, and postulates that this hormone is linked with the amount of caecotrophe produced by the mother. When prolactin production is inhibited, the amount of anal excrement produced diminishes, although the pheromone itself is still produced by the caecal bacteria, as evidenced by the attraction exerted by the caecal contents of virgin rats for the pups (Leon, 1974, Table 1 J, p. 443).

Leon makes the following statement:

> Therefore, maternal pheromone may only be maternal in the sense that lactating females alone secrete sufficient amounts of prolactin to induce excess caecotrophe defecation and thereby attract mobile young. (p. 447)

Taking the mechanism back two more stages, Leon postulates that suckling induces prolactin release in the mother, which in turn stimulates eating, bringing about an elevated food intake. The mother's increased intake occurs from parturition to Day 10, when it remains at a high level until weaning starts. This hypothesis was confirmed when it was shown that virgin females, after 16 days prolactin treatment ate more, and produced increased amounts of anal excrement which also attracted 16 day old young, when it was used as stimulus material in the olfactory discrimination apparatus.

Working on the pups' own capability to attract other young, Leon found that the attractive odour present in caecotrophe serves to mark the litter, besides the mother; and finally, when he has demonstrated, by using a non-toxic marker dye, that the young eat their mother's caecotrophe, he suggests that caecotrophe acts as a transitory weaning food for the pups.

(viii) Dietary Control of Maternal Pheromone in the Lactating Rat

Leon, 1975

Leon reports in this paper a series of experiments in which he seeks to investigate further the mechanism of pheromone production put forward in his monograph (1974). The elevated food intake displayed by the mothers, and which is associated with high prolactin levels, along with the quality of diet, come under scrutiny. He demonstrated that lactating females which were underfed failed to emit the pheromone, and, after feeding females from the fourteenth day of gestation on different diets, he found that rat pups were not attracted to the maternal pheromone emitted in the anal excrement of mothers fed on a different diet, and that this was also the case when they were given the choice of maternal odours with which they were unfamiliar.

> These data demonstrate a specificity of the pheromonal bond, since mothers emit maternal odors characteristic of their diet and individual litters respond to that odor and not to the odor of mothers eating another diet. (p. 315)

When mothers were given a diet containing sucrose as its only carbohydrate, and which inhibited the emission of caecotrophe and the maternal pheromone, the pups, when tested with the anal excreta obtained from a pheromone producing animal failed to show the normal responsiveness (Experiment 2, on quality of diet). Exposure to the odour emitted by a normal mother through being raised in the same colony room did, however, result in their having a preference for the maternal excrement. The importance of the pups' early experience calls for a drastic reappraisal of the maternal pheromone, and this is provided by Leon:

> Clearly, the pheromone is not an innate species-typic releaser, but the approach behavior appears to be an individually learned response on the part of the pups to the specific odor of the mother. Although a maternal pheromone may be emitted by all members of the species, the individual maternal odor is characteristic of the microbial action specific to each maternal diet. And although all mobile rat pups may be attracted to the odor of lactating mothers, their response is guided by the familiarity of that emitted odor. The form of the pheromonal response is therefore species-typic, but the pheromonal bond exists only in terms of individual experience.
>
> (p. 317)

An explanation in terms of conditioning follows. After receiving warmth and nourishment near the source of pheromone emission, the pheromone becomes a conditioned stimulus - bringing about the conditioned approach response. However, as the data indicate that pups prefer to approach the odour with which they are familiar, the attraction can also be explained in terms of imprinting. Ten years previously, researchers had shown that exposure of young rats and mice to an artificial odour during early life resulted in a preference for animals emitting that odour in subsequent adult social and reproductive interactions, this being an instance of olfactory imprinting (Mainardi <u>et al</u>., 1965; Marr & Gardner, 1965; Marr and Lilliston, 1969).

Leon concludes that the mechanism by which the maternal pheromone exerts its influence could be either a "non-specific familiarisation process", associative learning, or imprinting; which one is yet to be determined. He suggests that behavioural bioassay should continue to be used as the method of choice in identifying the individual maternal odours, as "....the chemical identification of the maternal pheromone characteristic of each maternal diet would appear to be an uneconomic undertaking.".

PART III

The Maternal Pheromone in Small Mammals

Between 1970 and 1976 the growing awareness of the importance of olfaction was reflected in the increasing number of published papers devoted to various aspects of this topic.

Typical studies are those by Stern (1970), Carr (1973), Aron and Chateau (1971), in which the role of odour in reproductive behaviour in rats is examined. Thompson and Edwards(1972) and Rowe and Edwards (1972), showed that olfactory bulb removal adversely affects mating behaviour in mice; Gandelman et al. (1972) looked at the role of olfactory cues in maternal behaviour in mice, using centrally induced anosmia, whilst Fleming and Rosenblatt (1974) undertook the same kind of work with rats. Alberts and Galef (1971) first described a useful technique they developed to induce anosmia by a peripheral method, later Alberts wrote a theoretical review on experimental olfactory deficits (Alberts, 1974). The differences between rats and mice, with respect to reproductive behaviour were now becoming apparent; on the whole it seems that mice are more dependent than rats upon their sense of smell. A review summarising the ontogony of social olfaction in vertebrates, in which pheromones figure, is provided by Cheal (1975); this supplements an earlier, more general review of the role of olfaction in animal behaviour (Cheal and Sprott, 1971).

There are no papers in which the maternal pheromone in another strain of rat is investigated, but there are a few reports of work carried out on other species in which the maternal pheromone - or at least, olfactory-guided behaviour, is mentioned. Falling into the latter category comes the work on the golden hamster by Gregory (1974), Gregory and Bishop (1975), and Devor and Schneider (1974); gerbils were investigated by Wallace _et al._ (1975); guinea pigs by Porter and various colleagues (Porter, Fullerton and Berryman, 1973); rabbits by Mykytowycz and Ward (1971); the spiny mouse by Porter and Ruttle (1975), and kittens by Rosenblatt (1972).

Golden hamsters showed a strong preference for home cage bedding obtained from the cage of an adult male, and also from a non-pregnant female, when both were compared with fresh bedding, between the ages of 7/8 and 11 days. However, they did not discriminate between home cage bedding and that obtained from a lactating female (Devor and Schneider, 1974); the investigators concluded that a single pheromone may be emitted by all adult hamsters, with a greater concentration given off by the lactating female. Gregory and Bishop (1975) undertook a more detailed exploration of the hamster odour preferences; they too found the choice of home cage odour starting to be displayed at about

7 days age, but extending to 16 days. Although this preference synchronises very well with the diminution of retrieving behaviour which occurs (in a similar manner to the rat), in fact Gregory and Bishop failed to demonstrate that the pups had a specific preferential response for the mother, and they do not confirm the existence of the maternal pheromone in the hamster. They also express some dissatisfaction with the behavioural measures used in these studies, which they describe as "relatively gross", and suggest that investigations of a neurological nature would be more appropriate.

Work carried out on the gerbil suggests that the young of this species prefer home cage shavings to those obtained from strange females, or clean shavings, and that they do not discriminate between shavings from lactating females' cages and those from their own (Wallace, Thiessen and Issacks, 1975).

Preliminary investigations on guinea pigs showed that they remain near their mother or another lactating female (Porter et al., 1973), and it has also been found that young kittens will use olfactory cues associated with the home cage to return there after they have been placed at some distance from it (Rosenblatt et al., 1969; Rosenblatt, 1972).

Mykytowycz and Ward (1971) suggested that wild rabbit nestlings may be responding to a maternal pheromone when they showed marked behavioural changes in response to swabs infiltrated with urine, anal gland or inguinal gland secretions.

Although many researchers were investigating various aspects of olfactory phenomenon in mice at this time, no work on the maternal pheromone in this species was published before 1976.

PART IV

The Status of the Maternal Pheromone after Five Years' Research

Although research indicated that olfaction plays a role in the mother-young relationship in a number of small mammals, the only substantial and systematic investigations into the maternal pheromone were the ones carried out on the Wistar strain of rat by Leon and Moltz and their colleagues.

After having demonstrated that 12 - 21 day old rat pups were attracted towards an odour emanating from a lactating female rat, and naming the cue involved the "maternal pheromone", they set about assessing various parameters, using behavioural bioassay. Leon's move to a separate laboratory resulted in two distinct lines of research emerging by 1975. Moltz and his colleagues were pursuing the traditional endocrine approach, and meeting with some success in explaining maternal pheromone emission in terms of an elevated prolactin level model. But Leon, after having demonstrated that high prolactin levels, elevated food intake, and caecal bacteria are necessary for the emission of the pheromone, found that after various dietary manipulations, young pups were attracted to an odour with which they were familiar, rather than a single specific substrate. He finally advanced three possible mechanisms for the "pheromonal bond"; a non-specific familiarisation process, associative learning, or olfactory imprinting, and suggested that the maternal pheromone, whose parameters had been delineated with such early assurance, may not be a single substance at all, but perhaps one of several possible attractants emitted by lactating female rats.

Despite Leon's change of position, the term "pheromone" remains, carrying the implication of releasing a rather stereotyped behaviour pattern in the recipient.

Although before 1976 there were no publications on the maternal pheromone in the rat other than those written by Leon and Moltz and their colleagues, the findings of approach behaviour based on olfactory cues in young of several other small mammalian species served to strengthen the concept of the maternal pheromone.

CHAPTER III

A REPLICATION OF LEON AND MOLTZ'S EARLY WORK ON ODOUR PREFERENCE IN YOUNG RATS

INTRODUCTION

Starting in January, 1976, work was undertaken in the Psychology Department at Hull University to investigate the precise nature and role of the maternal pheromone in the laboratory rat. As a starting point, it was desirable to replicate the original finding by Leon and Moltz (1971), and confirm that an attractant was emitted by lactating female rats.

For this replication, and most of the experiments reported in this thesis, the apparatus, subjects and test procedures are constant. Consequently, the information relating to the basic method will be supplied only once, in the section on general methodology which comprises Part I of this chapter. Because much of the subsequent experimental work involves manipulations of extremely basic variables, of the type described in Part I, any deviations from the standard procedure for a particular experiment will be indicated in the individual report on that experiment.

After the olfactory discrimination apparatus had been built, the first experiment was conducted and the outcome is reported in Part II of this chapter. Part III comprises a discussion of the results and problems arising from the replication.

PART I

General Methodology

Animals Used in the Experimental Work

(i) Strain

Three strains of *Rattus norvegicus* were used in the investigations into the maternal pheromone.

In all the early experimental work, the strain of rat used was the black-hooded PVG/C rat, originally obtained by the Psychology Department, Hull University, from the M.R.C. Unit at Carsholton in 1965. This inbred strain (F-52 in 1965) had been derived from WAG/g at F-13 by Glaxo in 1947, but since becoming the standard colony animals in the Hull Psychology Department they were maintained on an outbreeding programme within the confines of a random mating schedule.

In later experimental work inbred Wistar rats were used, and these were obtained from a firm of commercial breeders, Messrs. Bantin and Kingman Ltd., Aldbrough, Nr. Hull. Throughout the 4 generations which were bred after the purchase of the original animals, a strict inbreeding programme was adhered to.

In Experiment 15, Sprague-Dawley rats obtained from stock animals housed in the colony kept by the Zoology Department, University of Hull, were used. These outbred Sprague-Dawley rats also came from stock which had originally been purchased from Messrs. Bantin and Kingman Ltd., Hull.

(ii) Maintenance

All the experimental data were derived from male and nulliparous female rats aged 130 - 150 days; nulliparous female rats which had been mated at 90 ± 10 days, and their pups.

When breeding was to be carried out, males were assigned to females on a one to two basis, and they remained in the females' cages for one week. Pregnancy was determined subsequently by weekly weighing of the females.

At least one week before the expected date of parturition, females were separated from each other and housed singly in opaque white polythene cages, 36 x 16 x 15 cm, where food and water were available on an ad lib. schedule. At the time of separation, females were provided with approximately 50 gm shredded paper for nesting material, and they were then left undisturbed apart from when the litter was culled and removed for experimental testing. Two days before the expected date of parturition (the 19th day of gestation), cages were inspected daily at 9.00, 12.00 and 18.00 hours for births. The day of birth was designated Day 0.

The diets used throughout the experiments varied, and so the particular diet given during any one experiment is indicated in its procedural section.

The following diets were used:

41B (Modified) Oxoid Laboratory Animal Diet

Labsure Diet CRM

Labsure Diet PRD

The Bradshaw Rat and Mouse Breeder Diet

Details of the composition of the diets used are given in Appendix I, with the exception of the Oxoid diet. The manufacturers of this diet, Herbert Styles (Bewdley) Ltd., would not supply details of its constituents.

The lighting schedule in the main colony holding room (where the recently mated female rats were housed until after their litters had been used for experimental work), and testing room was a normal daylight one with the light period between 6.00 and 22.00 hours. The sources of illumination were fluorescent lighting strips and a 150 watt bulb.

The temperature of these rooms was kept between 17° and 24° C.

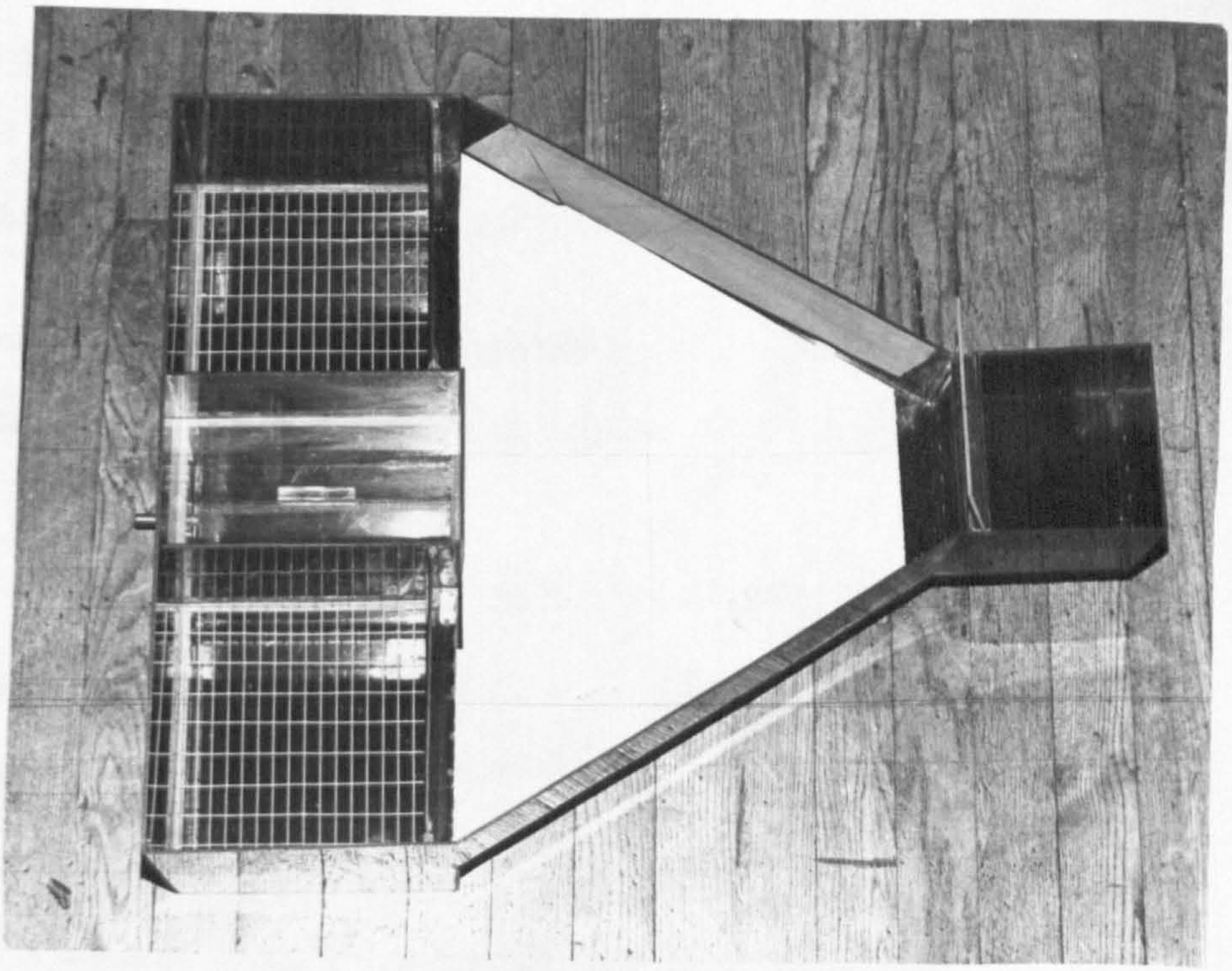
Apparatus

(i) The Olfactory Discrimination Apparatus

The olfactory discrimination apparatus was made as far as possible to the specifications given in Figure 1 (Moltz, 1975), and is shown in Plate 1. As mentioned in Chapter II, some of the details needed for the construction of an exact copy were inadequate or missing, with the result that the apparatus was not completely identical to the original. The differences between the apparatus built in Hull, and that used by the Americans are summarised at the end of this section.

The apparatus comprises a triangular open-field with a start box 15 x 15 x 13 cm at its apex, facing 2 goal boxes, each 23 x 23 x 28 cm. The sides were constructed from black plexiglass, 13 cm high, and the removable tightly fitting top was transparent. The goal box tops were covered with thick brown paper so that the goal box interiors

Plate 1



The olfactory discrimination apparatus. The picture is taken from directly above the apparatus; the start box lid is open, and the cover for the whole apparatus is not in place.

were dark.

Forced air entered the goal boxes and flowed towards the start box at a rate of 29 litres/min. The small motor driving the airflow pump was placed on the floor below the table on which the apparatus stood, where it created a constant masking noise of 65 dB. The apparatus was aligned to a fluorescent light strip in such a way that the open-field and the two goal box doors were illuminated with equal intensity. This was checked with a spot photometer.

The differences known to exist between the Hull apparatus and that used by Leon and Moltz are listed below:

1. A difference of 5 cm^2 in the area of the start box floor.
2. Plain paper sheets, rather than polythene-backed sheets were placed on the arena floor during testing.
3. The rate of airflow was 29 litres/min in the Hull apparatus as compared with 55 litres/min.
4. The motor driving the pump was a little quieter at 65 dB, than that used by Leon and Moltz, which created a constant noise of 72 dB.

<u>(ii) Cages used for Excrement Collection</u>

Two types of cage were used to collect anal excrement and/or urine from adult rats.

The first was a metabolic cage (20 x 30 x 15 cm) in which the animal is housed on a wire grid through which faeces and urine fall for collection. An inverted funnel arrangement, like the one described by Jones, Dilks and Nowell (1973), ensures that faeces are not collected in the same place as the urine, so that the two do not become mixed whilst awaiting removal. Contamination of one by the other is unavoidable however, as the urine often comes into contact with faecal matter on its passage down to the collecting bottle.

A simpler method of collecting anal excrement was to use small holding cages (20 x 35 x 15 cm) which had grid floors. The grid was approximately 2 cm above a paper-lined collecting tray. Although the paper floor soaked up the urine which was secreted by the animal, there was more cross-contamination occurring than when the metabolic cages described above were used.

The Standard Test Procedure.

Testing of pups in the olfactory discrimination apparatus took place between 9.00 and 17.00 hours, after pups had been deprived of maternal attention by removing the mother from the home cage for a period of 3 hours.

Pups were run in the apparatus singly, and each pup was tested only once. The age of the pups at testing was 16 - 24 days. Tests were carried out in a room separate from that in which the animals were housed; the pups were taken to the testing room in their home cages 30 - 60 minutes before they were tested.

Testing comprised placing a pup immediately in front of the closed start box door and allowing it 10 minutes in which to make a choice of goal box - the criterion being that it had to descend right into the goal compartment. After a pup had been run the paper sheet covering the floor of the apparatus was changed, and when half a particular litter (normally 3 pups) had been run, the whole apparatus was washed with warm water, and the goal box contents exchanged. The presentation of goal box contents was such that a particular arrangement appeared first for the first half of a litter being tested, on alternate occasions. This counterbalancing procedure was designed to spread any confounding variables which might be present (e.g. due to lighting effects, alignment of the apparatus, etc) across all testing conditions.

Any deviations from this procedure, which will be referred to as the standard test procedure, will be noted in the procedural sections of the particular experiments in which they occur.

PART II

EXPERIMENT 1

Purpose

The purpose of this experiment was to confirm that rat pups aged 18 days are attracted either to a lactating female, or to some substance present in her anal excrement, and that this would be in preference to a non-lactating female, or her faeces. These two sets of stimulus material, opposing live animals, or anal excrement are treated separately in Parts a) and b) of this experiment.

The experimental hypothesis was that 18 day rat pups would approach the lactating female or her anal excrement, rather than the stimulus material from the non-lactating female. As an attractant should elicit clear approach tendencies if it is to be regarded as pheromonal, it was decided that a minimum of 75% of the pups tested would have to enter the maternal goal box if the experimental hypothesis was to be confirmed. With the number of pups used in this experiment (16 in both parts), this would give a one-tailed probability of 0.038 with the Binomial Test.

Apparatus

The olfactory discrimination apparatus was used.

Holding cages (20 x 35 x 15 cm) with paper-lined floors were used to collect anal excrement for 3 hours before testing began. Food and water were available in these cages during this period.

Subjects

Thirty-two 18 day old rat pups were used. They were obtained from 4 litters born to strain PVG/C females which had been mated at 100 ± 10 days. The litters were culled to 8 pups on Day 0.

Two virgin female rats were used in this experiment, and were of the same age as the maternal animals, i.e. 138 ± 10 days when testing took place.

Maintenance was as described in Part I of this chapter, and the animals were fed on the Oxoid 41B diet.

Procedure

a) Choice of live animals in the goal compartments.

Using the standard test procedure, with the exception that the pups were placed in the start box, rather than immediately in front of its door, pups were tested in the olfactory discrimination apparatus after 3 hours maternal deprivation. They were allowed 10 minutes

minutes to make a choice between their own mother and a lactating female present in the two goal boxes.

b) Anal excrement as the stimulus material.

The intention was to take samples of anal excrement which had collected in the holding cages during the 3 hour period from both the lactating and non-lactating females, and to place roughly equal quantities of each in the two goal boxes. Leon (1974) reported collecting anal excrement in this manner, taking it off the holding cage paper-lined floor by means of clean glass slides.

Results

a) Mothers versus non-lactating females.

All 16 pups tested chose the goal compartment containing the lactating female (their own mother) in preference to that occupied by the non-lactating female.

b) Relative attraction of excrement samples.

It was not possible to compare samples of anal excrement in the goal compartments. During their 3 hours sojourn in the holding cages, the lactating female rats had not been idle. Displaying characteristic maternal behaviour, they had rapidly torn up all the paper flooring in the cage and reduced it, together with their abundant anal excrement and urine to an inseparable pulp. In contrast to this, the non-lactating females left their cage floor lining intact, but failed to excrete any faecal matter during the 3 hour period.

PART III

DISCUSSION OF RESULTS OBTAINED IN EXPERIMENT 1

Comparison of anal excrement

Procedural difficulties, only apparent at the time of excrement collection prevented the testing of pups' preferences as planned. Clearly the method of excrement collection used by Leon and Moltz needed modification before another attempt at replication could be made. It was decided that the problem could be overcome by lifting the holding cage above the level of the collecting tray, and separating the two by a grid floor, as described in Part I of this chapter. The next experiment incorporates this modification.

Another modification which was retained after this experiment was to place the pups immediately infront of the start box door, rather than inside the start box. It was discovered that pups placed in the start box preferred not to leave it after the door was opened, but to remain inside the start box, going to sleep after about 5 minutes. In future experiments, the 10 minute period allowed for pups to make their choice began when the experimenter replaced the apparatus cover after having put the pup into the arena.

Preferences exhibited for live animals in the goal boxes

It would be extremely pleasing to be able to report the overwhelming choice of the mother by the rat pups as evidence in support of the existence and potency of the maternal pheromone. Unfortunately this cannot be done, as when the pups were given a choice of either their own mother or a non-lactating female in the goal boxes, two important confounding variables became apparent.

First, the lactating female was extremely active, and constantly moved around in the goal box - thus emitting auditory cues, besides olfactory ones. It was not known why the mother should do this, particularly as lactating females tend to show a general reduction, rather than increase in movement, but her restless agitation was very marked when a pup was in the arena, thus suggesting that it was emitting either auditory or odouriferous cues to which she was responding. In contrast to this, the non-lactating female went to sleep in the goal-box, or else investigated, with what seemed like mild curiosity, the wall of the compartment adjacent to the other goal box, through which constant scuffling and thumping noises were audible.

That pups will approach a source of noise is known (Bolles and Woods, 1964); recently the role of ultrasound in mother-infant interaction has been investigated (Sewell, 1967, 1968, 1970; Noirot, 1970; Allin and Banks, 1972; Smith, 1975), the evidence to date suggesting that it may have quite an important function. Whilst the olfactory discrimination apparatus prevents the mother and pup from actually seeing each other, it allows auditory variables to be confounded with olfactory cues.

When the pups approached the goal compartment from which sounds audible to humans, and possibly ultrasound, were emerging, the second fault was revealed. As soon as the pup approached the goal box ledge, and put so much as a whisker or tail through the gap at the bottom of the swing door, the mother would instantly seize the appendage and pull the pup into the compartment. This retrieving behaviour was only shown by the lactating female, and so constitutes another confounding variable.

Conclusions

It can be seen then, that what was given as "choice of the maternal compartment", according to Leon and Moltz's criterion, was perhaps no more than the pups' movement towards a source of noise and odour, coupled with normal retrieving behaviour on the part of the mother. What at first sight had appeared to be a simple replication, resulted in the collection of data which, whilst failing to say much about the maternal pheromone, did bring to light several factors worthy of consideration.

First, but perhaps least important, were the technical and procedural details which needed modification. It would be necessary to amend the excrement collection technique so that faeces could be collected and weighed, rather than being inextricably mixed with paper pulp and urine. Evidently the non-lactating females would have to be kept in collecting cages for a much longer period of time is samples of anal excrement of anything like the quantity excreted by the mother were to be obtained. In his monograph (1974), in which he used excrement in the goal boxes, Leon fails to mention what amount he collected, and if, when excrement obtained from the mother is matched with non-lactating females' faeces (Leon, 1974, Experiment 1F), whether the two amounts are equal. If the amounts were not equal, then this creates a confounding variable in the experiments in which excrement is used as stimulus material.

Differences in the behaviour of both the maternal rats and their pups, used in the laboratory at Hull were evident. The mothers' display of retrieving and nest-building activities affected both the collection of excrement and the interpretation of results (as recounted above);

the pups' reluctance to leave the start box was also a difference and slight difficulty. The easiest explanation for this difference is that the pups used in the Hull laboratory had a different "temperament" - this perhaps being determined by their strain, which differed from that used by Leon and Moltz.

Finally, a point which became obvious during excrement collection, but which is never mentioned by Leon or Moltz, is the difficulty, and what turns out to be an impossibility, of complete separation of urine and faeces if they are eliminated by the animal in the normal manner.

In the early work on the maternal pheromone, when its existence was being demonstrated, the question of whether the attractant was present in urine or faeces was relatively unimportant. In the first experiment where Leon and Moltz test the attractive properties of excrement or secretions, rather than using a live animal (Leon and Moltz, 1971), the goal box was soiled by the mother before testing took place, and without doubt, urine and faeces would both be present. They would also both be present, and mixed to a certain extent, in the procedure described by Leon (Leon, 1974), where the animal is isolated in the collecting cage with the paper floor, although much of the urine would be absorbed by the paper. In his later work, Leon attempts to separate urine and faeces by using metabolic cages. Whilst this method appears to be adequate in this respect, and was adopted for much of the experimental work undertaken in Hull, there is no type of metabolic cage available where urine does not at some stage run down past the boli before it is collected in a separate container. Thus contamination of faecal matter by urine, and urine by faeces can occur.

The only occasions when Leon and Moltz successfully separate the two products is when they take the contents of the caecum directly from a recently killed animal. For the present, it is sufficient to recall that in dealing with pheromonal agents, one is up against a situation where, quite literally, molecules of a substance can exert an infleunce. In any work on the locus of emission on the maternal pheromone, avoidance of mixing the secretions and excretions is paramount. It may be that contamination during excrement collection is the cause of the different claims made by Nyakas and Endröczi (1970) and Leon (1974). The former researchers claim that rat pups were attracted to urine, whilst the latter claims that it does not contain an attractant.

The most serious implication of Experiment 1 is yet to be considered. This is the gross inadequacy of the experimental paradigm used by Leon and Moltz, in which live, mobile animals are used in goal compartments. This choice of stimulus material is used extensively by the Americans, and in particular throughout the work in which they map out the parameters of maternal behaviour (Leon and Moltz, 1972; Moltz and Leon, 1973; Leon and Moltz, 1973; Moltz, Leidahl and Rowland, 1974; Leidahl and Moltz, 1975).

The confounding variable of noise, either from an active mother moving about in reponse to ultrasonic calling, or odour, from her pup in the apparatus, plus the fact that she is likely to pull an approaching pup into the goal box with her, means that this experimental design is completely unsound. For this reason, it was decided not to use live, mobile animals as choice stimuli in any subsequent experimental work with the olfactory discrimination apparatus.

The conclusions drawn by Leon and Moltz and their colleagues about the maternal pheromone, cannot be treated as valid whenever they are based on work in which live animals have provided confounding variables. Chapter XIV comprises a reappraisal of the North American work on the maternal pheromone, in the light of this, and other experimental findings reported in this thesis.

CHAPTER IV

EXPERIMENT 2: A SECOND ATTEMPT TO DETERMINE WHETHER THE MATERNAL PHEROMONE IS PRESENT IN THE ANAL EXCREMENT OF LACTATING FEMALE RATS

INTRODUCTION

It was learned from Experiment 1 that if mobile animals were used in the goal compartments of the olfactory discrimination apparatus, confounding variables were present; also, that excrement could not be collected satisfactorily in the manner described by Leon (1974). In Experiment 2, anal excrement only was to be used as stimulus material for comparison, and it would be collected in a different way, using the holding cage method described in Chapter III. In order to place roughly equal amounts of excrement in the goal boxes, more faeces had to be obtained from the non-lactating females than in the first experiment. This was done by using several virgin females at once, and by keeping them in holding cages for twice as long as the mothers.

Chapter IV comprises the purpose, procedure, results and conclusions of the second experiment, which was an attempt to discover whether the maternal pheromone is present in the anal excrement of lactating female rats.

EXPERIMENT 2

Purpose

The purpose of this experiment was to determine whether 17 day old rat pups would be attracted towards the excrement obtained from their own mother, rather than that obtained from a non-lactating female. If they were, then this would be adduced as evidence that an attractant, the maternal pheromone, was present in the maternal faeces.

On the basis of Leon and Moltz's findings, the experimental hypothesis established was that rat pups would prefer the maternal to the non-maternal excrement; the Null Hypothesis being that the division into two would not be one other than might be expected by chance. With $\alpha = 0.01$, 90 of the 151 pups to be tested (60%), would have to enter the maternal goal box before the Null Hypothesis could be rejected. (Binomial Test, one-tailed prediction.)

Subjects

One hundred and fifty-one 17 day old rat pups were used. They were obtained from 19 litters born to strain PVG/C females which had been mated at 100 $\pm$ 10 days. All litters were culled to 8 on Day 0; one litter comprised 7 pups.

Anal excrement was collected from 3 nulliparous females of the same strain and age as the 19 mothers.

Maintenance was as described in Chapter III, Part I; animals were fed on the Oxoid 41B diet.

Apparatus

The olfactory discrimination apparatus and holding cages with grid floors, described in Chapter III, Part I, were used.

Procedure

Before testing started, faecal matter was collected from the mothers of the litters to be tested on that particular day, and 3 of the nulliparous females, by isolating them in the holding cages with grid floors above a paper-lined collecting tray. Mothers were kept for 3 hours in these cages, and the non-lactating females for 6 hours. All the animals were housed individually in these cages.

Immediately before the testing session began, faecal matter from the maternal and non-lactating females was taken from the collecting cages, and similar amounts of both types (between 3 - 5 gm), were placed on two very fine wire mesh trays, these then being placed inside the two goal box compartments.

The pups were then tested, using the standard test procedure.

Results

The results of Experiment 2 are given in Table 2.

Table 2

Choice behaviour of 17 day old rat pups: preferences for anal excrement obtained from the mother or a non-lactating female

PUPS' BEHAVIOUR			
Chose maternal excrement	Chose non-lactating females' excrement	Did not enter a goal box	Total
79 (52.3%)	47 (31.1%)	25 (16.6%)	151

As fewer than 90 pups entered the maternal goal box, the experimental hypothesis was not supported. The exact probability of obtaining 52.3% entries in one of the two goal boxes is 0.34 (Binomial Test; one-tailed prediction).

Discussion

After the procedural difficulties encountered in the first experiment had been overcome, it was disappointing to find that there was no evidence of the presence of an attractant pheromone in the maternal excrement. Interpretation of the results was not made simple though, by a number of pups failing to enter a goal box. Of those that did, 79 (62%) chose the maternal excrement, thus indicating a preference.

Problems of data analysis and interpretation will be examined in Sections (ii) and (iii) of this Discussion, following a consideration of some aspects of the experimental work which might have caused the failure to obtain the expected results.

(i) Variables considered likely to have caused the failure to replicate

It seemed reasonable, at this stage of the investigation, to assume that the maternal pheromone did exist, but that some variable which was apparently of importance had been overlooked, and that it was masking or obliterating its attractant powers in some way. Consequently, it was necessary to examine all the procedural details closely; ascertain which ones differed from those reported by the Americans, and alter them so that the next attempted replication was identical, as far as possible.

The following differences were identified:

1. Strain of rat used

Leon and Moltz had used Wistar rats, whilst the ones used in this experiment were of a hooded strain. It could be possible that the maternal pheromone is strain specific.

2. Size of litter

Leon and Moltz worked with litters which had been culled to 6 at birth.

3. Diet

The American rats were all fed on Purina rat chow. The rats in the Hull laboratory were fed the Oxoid diet.

4. Apparatus

The differences of size, material and airflow listed in Part I of Chapter III were considered. The slight difference in size, and the type of floor covering used were not thought to be important, but the rate of airflow could be crucial, if it were either too gentle or too strong.

The most likely cause of failure, and easiest to rectify, was considered to be the one arising from an apparatus deficit. Consequently, the rate of airflow was examined as the independent variable in Experiment 4. The size of litters could easily be altered to 6 pups, and this was done for subsequent work.

Strain and diet were less easily changed, and it was decided to examine these only if the alterations described above failed to bring about a successful replication. Meanwhile, the manufacturers of Purina Rat Chow and the Oxoid diet were approached for details of the composition of their diets.

(ii) Analysis of results

At first sight, the interpretation of the quantitative data obtained from experiments using the olfactory discrimination apparatus appears to be a simple matter. When most of the rat pups make a goal box entry, as seems to be the case in most of the experiments reported by Leon and Moltz, there are few problems of statistical analysis. The choices of the two goal compartments made by the pups can be compared using a test of goodness-of-fit; the binomial test, or simple χ^2 test, with an a priori expectation of a 50/50 division comprising the Null Hypothesis.

As soon as the results from Experiment 2 were obtained, it became apparent that the data were not of a dichotomous nature, and that the establishment of a theoretical distribution with which the results could be matched posed something of a problem.

The difficulty lay in the large proportion (16.6%) of pups which had failed to enter a goal box. Although slightly more than half the total number of pups run had chosen the maternal excrement, and indeed, 62% of all the pups which entered either compartment, did this constitute evidence for either a preference, or attraction towards the mother's excreta?

No problems arise over the statistical analysis of the data, in that goodness-of-fit tests are appropriate, but the question is; how is a distribution for matching purposes (the Null Hypothesis) determined? It could be done on either theoretical or empirical grounds (Siegel, 1956), but the importance of the choice was not appreciated until the data from Experiment 2 were collected and considered.

Leon and Moltz found that the majority of their pups entered a goal compartment, and the few which did not, they labelled "no choice". If one were to disregard the "non-choosers" in Experiment 2, then a simple dichotomy would exist. However, one must ask whether this approach can be justified. Leon and Moltz's practice of labelling the pups which remain in the arena as making "no choice" has resulted in an inclination to regard these animals as being of little significance, with the consequence that they can be discounted in statistical analysis. However, these pups could be considered as making a choice, namely to remain in the arena, and so they constitute an important third category.

Their failure to enter the maternal compartment could be construed as evidence against the existence of an attractant, and on these grounds it would be possible to group them with the ones which entered the non-maternal goal box for comparison purposes - reducing the analysis to a dichotomy once more. Alternatively, it might be more appropriate to compare the results obtained with a distribution where the dependent variable is divided into three categories rather than two - the expected values of each cell being 33.3% of the total number of pups run. This theoretical distribution is the equivalent of the first theoretical 50/50 dichotomy, the χ^2 test rather than the binomial test being the appropriate statistical analysis in this case.

Thus three approaches to data analysis have been generated, and it is instructive to re-examine the data obtained in Experiment 2 in the different ways outlined. These are:-

1. Division into two categories: choice of maternal excrement versus choice of nulliparous excrement. "No choice" pups are eliminated from the analysis.

2. Division into two categories: choice of maternal excrement versus the remainder.

3. Division into three categories; pups choosing the maternal excrement, choice of nulliparous females' excrement, and those remaining in the arena.

When the data from Experiment 2 were reanalysed in these three different ways, three values of χ^2 were obtained, and these are shown in Tables 3, 4 and 5.

Table 3

Analysis of data obtained in Experiment 2, using χ^2 test, and a division into two categories, with the "no-choice" pups eliminated

FREQUENCIES	CATEGORIES OF DEPENDENT VARIABLE		TOTAL
	Chose maternal excrement	Chose nulliparous excrement	
Obtained	79	47	126
Expected	63	63	126
$\chi^2 = 4.13$; df = 1; $p<0.05$			

Table 4

Analysis of data obtained in Experiment 2, using χ^2 test, and a division into two categories, "no-choice" pups being grouped with those which chose the nulliparous females' excrement

FREQUENCIES	CATEGORIES OF DEPENDENT VARIABLE		TOTAL
	Chose maternal excrement	Did not choose maternal excrement	
Obtained	79	72	151
Expected	75	75	150
$\chi^2 = 0.16$; df = 1; non-significant			

Table 5

Analysis of data obtained in Experiment 2, using χ^2 test, and a division into three categories

FREQUENCIES	CATEGORIES OF DEPENDENT VARIABLE			TOTAL
	Chose maternal excrement	Chose nulliparous excrement	Remained in arena	
Obtained	79	47	25	151
Expected	50	50	50	150

$\chi^2 = 29.5$; df = 2; p 0.001

There are now three separate statistical "results"; significance at the 0.05 level, no significance, and significance at the 0.001 level. This rather broad range shows that the statistical decision as to how to handle the so-called "no-choice" pups can be seen to be a rather important one.

This decision depends upon the nature of the experimental hypothesis, i.e. the claims made by the investigator. As Leon and Moltz and their colleagues only infrequently encountered rat pups which failed to enter a compartment, the problem of how to treat "non-choosers" was not a central issue. However, their neglect of this topic does not mean that it is of no consequence, and it is worth considering the experimental hypotheses which might be supposed to underlie their position in some detail.

Throughout their work, Leon and Moltz rarely make explicit experimental predictions. As the results they present are, on the whole, reasonably clear-cut, no difficulties arising from data analysis are mentioned, and in most of their experimental results tables, significance levels are given without reference to their derivation. This means that for the χ^2 values reported, the reader does not know how a particular set of "expected" frequencies was obtained. In fact they should depend upon the theoretical premises of the investigation.

If one were making the fairly weak experimental prediction that rat pups "prefer" the excrement obtained from their mothers to that from non-lactating females, then it would be admissable to compare two categories of dependent variable, as in method 1 given above. If, however, one makes the assertion that maternal excrement contains a pheromone which acts as an attractant, in a very positive sense, then the second method of analysis is appropriate, for it must be seen that pups move towards the pheromone rather than do anything else. Whichever position is adopted, it is hard to justify the first method of analysis, in which the "non-choosers" are simply discounted.

Although in their early work, Leon and Moltz talk about <u>preferences</u> for maternal excrement (e.g. Leon and Moltz, 1972; Experiment 1, p. 683), in later work, the stronger position is adopted. At the start of the paper on dietary control Leon (1975), refers to an olfactory cue "attracting" mobile pups, and to pups "readily" approaching the lactating female. Frequent use of the term "pheromone" also implies the stronger position. The very definition of a pheromone as a substance which elicits a relatively invariant type of response - in this case movement towards an attractant, implies a stereotyped approach response which eliminates other responses. If the olfactory cue contained in the maternal excrement is to be called a pheromone, then it is important that it has got strong powers of attraction, and is not simply approached by the pups because, say, it is the more familiar cue.

Thus if a researcher were to claim that rat pups displayed a preference for a particular substance, then a different set of comparison numbers for a Null Hypothesis would be used than if the existence of a pheromone were propounded.

Although Leon and Moltz often presented data which did suggest that there was an overwhelming approach response - possibly an attraction to a pheromone, the fact that this type of response was not elicited in the pups tested in Experiment 2 meant that a decision had to be made about an appropriate type of statistical analysis. The most sensible solution to the problem of how to obtain comparison numbers for the Null Hypothesis seemed to be to obtain empirical data. If a control group of rat pups were to be tested in the empty apparatus, then their choices would provide a base-line against which the choices made when stimulus material was present could be evaluated.

Experiment 3 comprises such a control group, and the results are reported in the next chapter.

(iii) Comparison of Experiment 2 results with those reported by Leon and Moltz, and an examination of their data analyses

Experiment 2 was undertaken following the model of maternal attraction advanced by Leon and Moltz. They do not report any experiment in which young aged exactly 17 days were tested (usually they worked with 16 day pups), but as this age falls easily within the 14 - 21 day age, over which they claim the pheromone is released, and the young respond (Leon and Moltz, 1972), this was not regarded as an important difference.

In two of the experiments reported by Leon and Moltz, the conditions of testing and stimulus material used are so similar to Experiment 2 that a direct comparison of results may be made. One is in the very first paper (Leon and Moltz, 1971), in which it is reported that 83% of 16 day old pups chose the goal box soiled by a strange mother, as opposed to one previously occupied by a nulliparous female. Apparently all pups entered one of the two goal boxes, as there is no indication to the contrary. The statistical test used in the analysis of results is not given, but a probability of $p < 0.01$ is reported, and this would be obtained from a χ^2 test (without Yates' correction), the value of χ^2 obtained being 7.5, df = 1. This is using method 2 (given above); there are no problems here, in that there were no "non-choosers" to deal with. As more than two-thirds of the pups tested opted for the maternal compartment there is no good reason for quibbling over the statistical probability of these results.

In a later report (Leon, 1974; Experiment 1F, Table 1), more results for 16 day young, tested under similar conditions to those in Experiment 2 are given. The reader is informed that 70% of the pups chose the 16 day anal excreta obtained from the mother, whilst 7% chose virgin anal excreta, and 23% failed to make a choice, i.e. failed to enter one of the two compartments. Although Leon only ran 30 pups under this particular condition, he reports the proportions in percentages, quoting a probability level of $p < 0.05$, using the χ^2 test. As he does not say how he grouped his data for analysis (and it has been pointed out in the previous section that this matter is rather crucial), a complete reanalysis of his results was undertaken. It seemed that whichever method of the three outlined earlier was used on his data, none gave rise to a probability level of 0.05, and so a letter was sent to Leon, asking him how he had obtained his probability values.

His reply, dated 28. 5.76., contained the information that he took the number of rats failing to enter either goal compartment, divided it into two halves, and added this value to each of the numbers of goal box entries made. In his letter he admits that this is not an ideal way of handling and analysing the data; it is certainly a method which can only be used when the numbers of rat pups failing to enter goal compartments are small. (See Appendix II)

In several of the publications by Leon and his colleagues, there is no indication of how significance levels were obtained, other than naming the test of goodness-of-fit used. Neither are *a priori* predictions for particular experiments made. Not only is the reader left in the dark as to how comparison figures for the χ^2 test are obtained, but the position regarding non-choosers is never mentioned at all. On occasions the results which appear only indicate which pups chose the maternal stimulus, and there is no information about whether or not the remaining pups enter the other goal box. This has already been pointed out in the description of the comparison made in the first paper published by Leon and Moltz (1971), and it also happens in a few subsequent publications (Leidahl and Moltz, 1975, Figure 2; Moltz, Leidahl and Rowland, 1974, Tables 1 & 2).

In reporting results from experiments in which the olfactory discrimination apparatus is used, precise information on the behaviour of pups failing to enter the maternal goal box, and the way the categories are handled in subsequent statistical analysis should be indicated. The decision about how to categorise the data rests upon the nature of the experimental hypothesis, which should also be stated explicitly.

It has just been shown, using the results obtained from Experiment 2, that the division of the data for analysis comparisons affects the significance levels obtained, and hence the interpretation of results. Thus the importance of clear predictions and information about statistical treatment can be seen.

Although Leon and Moltz obtained fairly clear-cut results on the whole, there are occasions when there is no overwhelming choice of one of the stimuli present in the goal boxes. An example is seen in the experiment just examined, (Leon, 1974; 1F), in which 30% of the animals fail to enter the compartment containing maternal anal excrement. These data are a little different from those obtained in a comparable test in the first paper (Leon and Moltz, 1971), when 83% chose the maternal, rather than virgin excrement. Perhaps the amount of excrement present was a confounding variable, as undoubtedly, after equal periods for the mother and virgin female in the goal boxes, there would be more anal excrement left in the maternal goal box. In the later experiment, excrement was collected from animals kept in holding cages, and it is likely that an effort was made to equalise the two amounts placed in the apparatus, although this is not stated by the writer, and may not be the case.

Other instances of where the behaviour of "non-choosers" should be considered for purposes of analysis occur in the papers on the parameters of pheromone emission (Leon and Moltz, 1972; Moltz and Leon, 1973; Moltz, Leidahl and Rowland, 1974), and in the paper on concaveated females by Leidahl and Moltz (1975).

A high proportion of "non-choosers" (50%) is reported for 12 day old rat pups in Table 3 of the paper "The Development of the Pheromonal Bond in the Albino Rat" (Leon and Moltz, 1972). This is much higher than any other figure for "non-choosers" given in the Table, indeed all the 10 day old pups made a "choice", although this did not entail entry into a goal box, as the younger pups were given different choice criterion. The change which apparently occurred over a two day period could not be an artefact arising from the alteration of criterion for "choice", because it was the same for both groups. Only pups older than 12 days were given the more stringent criterion of actual entry into a goal box, to constitute a "choice".

In summary then, the reader is not given enough information about how comparison figures for χ^2 test are obtained, or even about what kind of a contingency table is drawn up. There are enough comparisons with results of an equivocal nature reported by Leon and Moltz and their colleagues to necessitate giving these details.

Conclusions

Several things were learned from the results obtained in Experiment 2.

First, a number of differences in procedure and subjects which might have given rise to the failure to replicate the maternal pheromone effect were noted. These were details of apparatus construction, the strain of rat used, the number of pups in each litter, and the diet used for the animals in the Hull colony.

Second, it was found that methods of excrement collection were not entirely satisfactory, and it was realised that the Americans omitted to mention the problems which they must also have encountered. It seems likely that the quantities of excrement present in some of the early work were not controlled, and so confounded the experimental findings.

Finally, examination of the results obtained in this experiment, and in work by Leon and Moltz, suggested that whilst comparison categories which could be set up for statistical analysis appeared to be an arbitrary choice, their selection was an important matter. Any comparisons should be made on the basis of an explicitly stated experimental hypothesis; if necessary, data for the Null Hypothesis being obtained from control groups. Leon and Moltz were criticised for omitting to give a clearly stated experimental hypothesis, and for failing to provide information about the comparison categories used in the χ^2 tests carried out (even when some of their results indicated that several pups failed to enter a goal box).

However, despite these considerations, and mainly because of the procedural differences noted above, the existence of the maternal pheromone was not seriously called into question.

CHAPTER V

THE ESTABLISHMENT OF A CONTROL GROUP

INTRODUCTION

Results obtained from Experiment 2 could be interpreted in three separate ways, according to whichever statistical comparison group was set up. An empirically established control group for comparison purposes would circumvent this problem.

In Part I of this chapter, the setting up of such a control group, and the results obtained from it are reported. In Part II, the results obtained from the previous experiment are reanalysed and discussed in the light of the new data.

PART I

EXPERIMENT 3: A CONTROL GROUP

Purpose

The aim of this experiment was to determine how rat pups would behave when they were placed in the olfactory discrimination apparatus without any stimulus material present in either of the goal compartments.

Apparatus

The olfactory discrimination apparatus.

Subjects

Thirty-six 17 day old rat pups obtained from 6 litters born to nulliparous strain PVG/C females which had been mated at 100 $\pm$ 10 days.

All litters were culled on Day 0 to 6 pups.

Procedure

The standard test procedure, using the olfactory discrimination apparatus was followed, and testing took place in 3 daily sessions between 12.00 and 17.00 hours.

Results

The data obtained from the control group are shown in Table 6.

Table 6

Choice behaviour of 17 day old rat pups: control group run in empty apparatus

PUPS' BEHAVIOUR			
Entered left-hand goal box	Entered right-hand goal box	Remained in the arena	Total
10 (27.8%)	9 (25%)	17 (47.2%)	36

Discussion

The results showed that even when there is no attractant present in the goal boxes, about half the pups can be expected to enter one or the other of them.

The division of the pups which entered goal boxes into two very similar sized groups indicates that there were no variables present in the apparatus - such as uneven lighting or airflow, which might affect the relative attractiveness of the two compartments and create a biasing effect.

As some of the pups entered goal compartments whilst others remained in the arena, it was concluded that on the whole, neither the goal boxes nor the arena constituted noxious stimulation which all pups would seek to avoid. However, the individual differences in the behaviour of the pups did suggest that for some, the arena constituted stress from which the darker goal box brought relief, whilst to others, the stress of being placed in the arena resulted in the pup exhibiting "freezing" behaviour, or darting forward very quickly. Some pups spent the entire testing time exploring the arena and making no attempt to leave it; in their case, the novelty of the situation did not seem to arouse fear. When individual reactions to the test procedure were recorded, four main types of response were observed:

1. Immediately on release the pup darted forward and entered a goal compartment.

2. The pup moved at walking speed to a goal box, where it might enter within about a minute, or remain in close proximity, but in the arena, for the test period.

3. The pup froze and remained immobile for the whole test session.

4. The pup explored the arena for the test session, and failed to enter a goal compartment.

It was rare for a pup to show more than one of these responses. Particular reactions did not seem to be litter specific, as siblings would often display very different reactions to the test procedure.

The similarity between the olfactory discrimination apparatus and the traditional open-field test became apparent after running this experiment. The open-field test provides stimulation to the pups which can result in very varied reactions, and although it has provided a popular means of quantifying rat behaviour, the interpretation of the various responses observed is exceedingly complex (Russell, 1972). It does not seem desirable to impose a test environment with poorly understood effects,upon behaviour in a simple discrimination task. At the time of running Experiment 3 though, it was supposed that if a maternal pheromone exists, and releases a specific approach response, then that response should be evident in apparatus such as the olfactory discrimination apparatus, and not unduly affected by variables present in the open-field situation.

The importance of the empirically established control group is perhaps only realised when the results obtained are equivocal. In none of the published work on the maternal pheromone is a control group, in which pups are tested in the apparatus with empty goal compartments, ever run. Whilst this might be reasonable for much of the work where clear-cut results are obtained, results from such a group would certainly help to clarify interpretation of some results reported (e.g. Leon, 1975, Experiments 2 and 3 on quality of diet).

After Experiment 3 had been carried out, the results obtained were used to provide a statistical comparison group for later experiments in which similar 17 day old PVG/C rat pups were used. [1] If any of the standard variables were altered, however, another suitable control group was run, so that for every experiment carried out, comparison data obtained from an appropriate group was available.

PART II

RE-EXAMINATION OF THE DATA OBTAINED IN EXPERIMENT 2

In the second experiment, it had been found that of the 151 pups tested, 79 (52%) chose to approach the mothers' excrement, 47 (31%) the non-lactating females' excrement, whilst 25 (17%) failed to enter a goal box. When the data were compared with various *a priori* hypotheses which could be made, it was found that their interpretation depended rather too much upon which hypothesis, and method of analysis was used.

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[1] More control groups were run under similar conditions a few months after Experiment 2 (Experiments 9 and 11). The almost identical results suggested that the pups' behaviour was stable over quite a period of time.

Using the results of Experiment 3, it was possible to carry out a comparison of the pups' choices with an empirically derived set of numbers; Table 7 shows both sets of data put into the form of a contingency table.

Table 7

Results obtained from Experiments 2 and 3

GROUP	PUPS' BEHAVIOUR			TOTAL
	Maternal OR Left-hand goal box	Non-maternal OR Right-hand goal box	Remained in arena	
Experiment 2 (choice of excrement)	79 (52.3%)	47 (31.1%)	25 (16.6%)	151
Experiment 3 (empty apparatus)	10 (27.8%)	9 (25%)	17 (47.2%)	36
$\chi = 16.46$; df = 2; p = <0.001				

In the χ^2 test, which gave highly significant results (p = <0.001), the control group entries into the right and left compartments were averaged to 9.5, so that the same figures were used for comparison against the maternal and non-maternal entries. As the right and left hand goal box contents were alternated during test sessions, their numbers can only properly be compared with the average goal box entries obtained when the apparatus is empty.

Conclusion

The interpretation of Experiment 2 was then, that although the pups were not overwhelmingly attracted to the maternal excrement, there was evidence of some attraction to this material. In the analysis, the three categories of behaviour observed were compared with three more sets of frequencies which were obtained from the pups themselves, rather than with a hypothetical set of three, divided evenly between each cell, as was done in the third method of analysis, Chapter IV. Although the outcome was the same (a difference with a significance level of 0.001), the comparison carried out in this chapter was based on real, rather than hypothetical events.

CHAPTER VI

FACTORS WHICH MIGHT PREVENT THE REPLICATION OF THE MATERNAL PHEROMONE EFFECT

INTRODUCTION

Part I of this chapter summarises the view of the experimental findings which was held by early summer 1976. In Part II, Experiment 4 examines the rate of airflow through the test apparatus. It was hoped that by manipulation of this variable, more positive results from pups tested in the olfactory discrimination apparatus would be obtained.

PART I

A CONSIDERATION OF THE PROBLEM

Experiments 1 and 2 alone said a great deal about the problems encountered in testing for the maternal pheromone, something about the character of the North American work, but very little about the pheromone itself. When the results from Experiment 2 were compared with the data obtained from a control group run for the purpose of obtaining numbers for comparison (Experiment 3), the conclusion was that the earlier data provided only limited evidence for the existence of the maternal pheromone. As many of the pups tested entered the compartment holding the maternal excrement, rather than the other compartment, or remaining in the arena, their choice could perhaps be better regarded as a "preference", rather than as being due to the attraction of a pheromone.

At this stage of the research, three broad hypotheses were advanced as to why a strong preference for the maternal excrement had not been displayed by the pups. These will be listed, and each then discussed in turn.

1. The pheromone was emitted, but differences in the construction of the apparatus, or the rate of airflow, meant that the pheromone was not reaching the start box end of the arena.

2. The pheromone was present, but because of some inadequacy on the pups' part, it was not eliciting the appropriate response.

3. The pheromone was either not being emitted, or was not being emitted in sufficient quantities to be detected by the pups.

<u>1. The pheromone was not reaching the pups because of an apparatus fault.</u>

The differences in construction between the Hull apparatus and that used by Leon and Moltz were considered, and it was decided that the slightly differing dimensions were unlikely to be important. Also, that the type of floor covering used, as it did not have a strong odour, should not affect the results. One difference between the Hull and American olfactory discrimination apparatus which could not be easily dismissed was the rate of airflow. A small motor had been fitted to drive the airflow pump, but initially, the rate of airflow had not been measured. The current could be felt as a very gently draught emerging from the start box ventilation holes, and although it was strong enough to carry cigarette smoke through the apparatus, it might not be strong enough for testing purposes. Measurement revealed that the airflow rate was only 29 litres/min; a figure well below the 55 litres/min given by Leon (1974). This variable was considered in detail in Experiment 4.

2. <u>The pheromone was present, but inadequacy on the pups' part meant that they were failing to respond to it.</u>

The existence of the maternal pheromone was put forward by Leon and Moltz because the pups they tested consistently entered the goal compartment holding the maternal female or her excrement. Entry into the goal box was the criterion adopted by them, with the exception of very young pups, which were only required to move a certain distance towards the source of odour (Leon and Moltz, 1972). It could be the case that in this replication, the pups were attracted towards the maternal goal box, but failed to actually enter. Several pups which had failed to make a "choice", had either frozen, or spent a considerable part of their testing time just outside one of the goal box doors. Although the unquantified observations of the pups' behaviour so far had not indicated that they preferred to stay outside the maternal goal box, it was felt that careful recording of their movements in the arena might indicate a consistent preference which had been missed up to now. It was possible that the pups could discriminate the maternal odour, but that a less stringent criterion would have to be adopted in order to demonstrate its existence.

It was decided to observe the pups' behaviour in the arena, and to quantify it by dividing the floor into three parts, and recording the length of time the pups spent in each, during a test session.

Pencil lines were drawn on the paper floors, giving two adjacent recatangular areas immediately in front of the goal box doors (size 15 x 30 & 21 cm), and a triangular area between these and the start box. A plan of the arena floor with its divisions marked out is given in Figure 2.

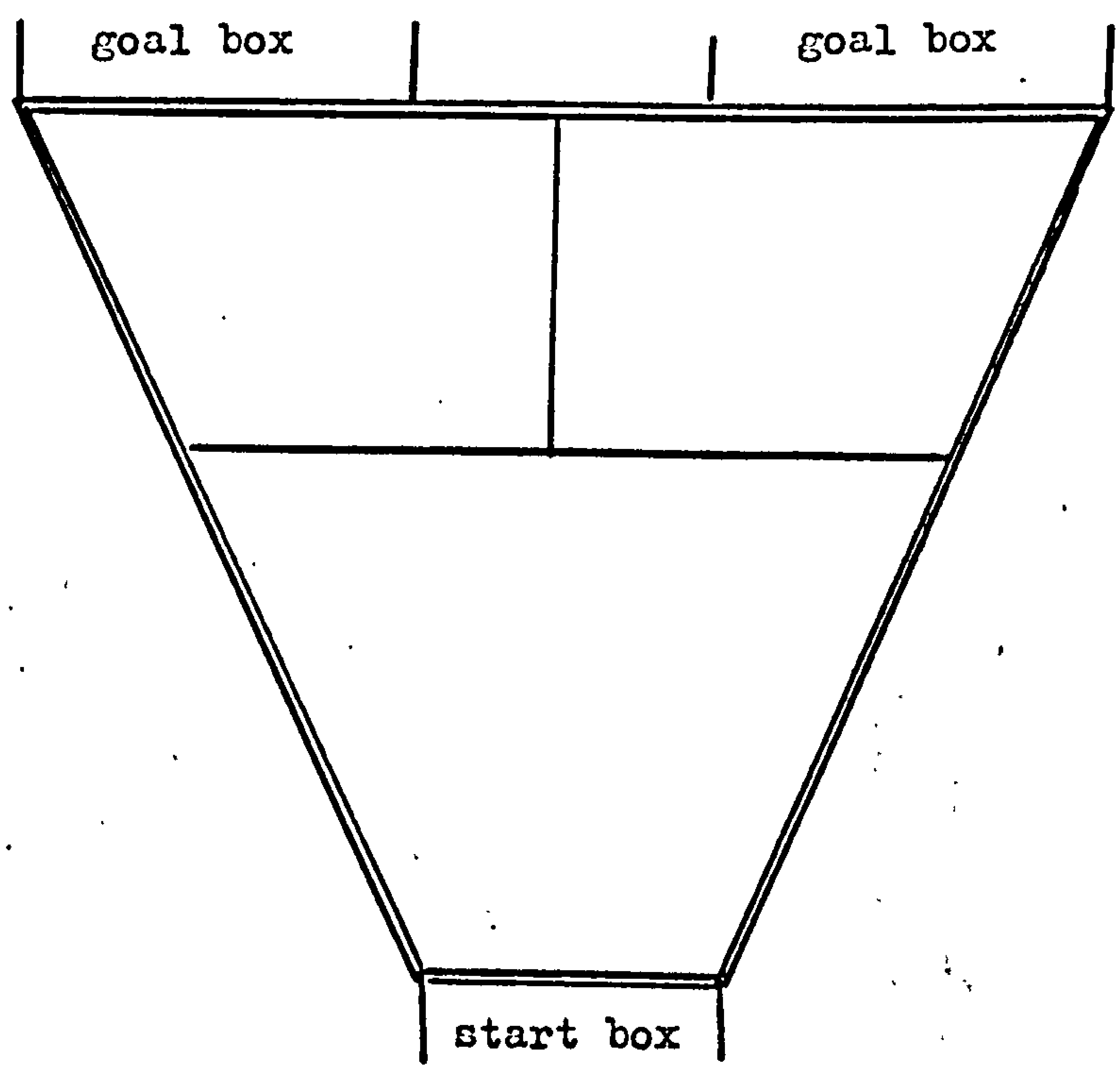


Figure 2: Plan of the arena floor, showing
the divisions marked out.

Whenever the olfactory discrimination apparatus was used in experimental work after this point, observation and recording of the pups' behaviour in the arena was carried out as a matter of course. The <u>time</u> a pup spent in each of the three parts was recorded, using two stopwatches, and the number of dividing lines crossed by a pup during its 10 min. testing period, an <u>ambulation</u> measure, was recorded.

Another type of "failure" on the pups' part was considered. They might be fully able to discriminate the odours present in the apparatus, but not be sufficiently "motivated" to approach the maternal odour. "Motivation", in the context of this work, simply refers to the pups' need for nourishment and warmth. If the pups were feeling relatively comfortable in these respects, then they may show less inclination to approach the mother. Presumably the pre-test deprivation period of 3 hours which Leon and Moltz used throughout their work reflects an attempt to manipulate motivation, by ensuring that the pups are in need of nourishment by the time they are tested.

A consideration of such motivation raises both practical and theoretical difficulties. Taking the latter first, if motivation levels are important, then the attractant involved must be regarded as a cue which is only approached by pups under certain conditions. It is doubtful whether this restriction on its ability to elicit a specific response would allow the cue to be regarded as a pheromone. From a practical point of view, motivation of pups to seek the source of a maternally-derived odour could be altered quite easily, by depriving pups of maternal attention for periods longer than 3 hours before testing took place. There is a danger here though, that young pups will become too weak, through loss of body heat and lack of nourishment, to move very far, and so will be unable to indicate through their movement towards a stimulus that they can make the discrimination, and respond appropriately. Pups older than about 13 days are able to withstand prolonged periods of maternal deprivation and not suffer such serious consequences. Manipulation of motivation level was undertaken with 18 day old rat pups in Experiment 9.

The pups' response to the test situation was examined in Experiment 11. It could be the case that many of the pups, whilst discriminating the odour, were so stressed by the open-field situation that they did not respond "normally", but showed darting or freezing behaviour. If rat pups were familiarised with the test procedure, then under less stress in the arena, they might show a goal box preference, in contrast to freezing or escaping from the open-field as rapidly as possible.

There was another possibility. This was that the rat pups used in these experiments lacked a good sense of smell - either because of ill-health (and the respiratory tract infection common to laboratory rats was a likely candidate here), or due to some deficit of a less pathological nature, a peculiarity of strain, for instance. It was important to establish that pups obtained from the Hull colony did have a sense of smell; after checking that they were free of any infection or disease, investigation into this matter was undertaken. This work comprises Experiment 5, and is reported in Chapter VII.

3. The pheromone was not being emitted by the mother, or else it was not present in sufficient quantities to attract the young.

At this stage in the proceedings, the explanation for the weaker experimental results obtained was considered more likely to be due to a diminished pheromone production on the mothers' part, rather than an absence of the pheromone.

Whilst being aware of the possibility that the pheromone might be strain specific or diet dependent, there was, as yet, no good reason for thinking that this was the case, and priorities were to establish that the pheromone was being carried through the apparatus adequately, and that the pups could smell. However, even though experimental work on the variables of diet and strain was not undertaken at this stage, it could fairly easily be ascertained whether the requirements named by Leon and Moltz as being necessary for pheromone production were met.

In his papers on dietary aspects of the pheromone (1974, 1975), Leon finds that three things are necessary for pheromone production and emission. First, the animals must have an elevated food intake; second, the diet must contain "raw carbohydrate" which provides the material for the growth of bacteria in the caecum; third, the diet must not contain antibiotic which would destroy the bacteria.

Although the manufacturers of the Oxoid diet, Herbert Styles (Bewdley) Ltd., were not prepared to disclose its composition, a spokesman who was given information about the nature of the problem stated over the telephone that the diet contains raw carbohydrate and does not contain any antibiotics.

Another possibility was that the maternal females were not consuming enough food to bring about the excess caecotrophe production. The intake of a group of lactating rats was measured daily from parturition to Day 21, when pups were removed from the mother, and the food consumption of these animals is shown in Figure 3. It can be seen that the mean intake rose from a pre-parturitional level of 13 gm to 48 gm on Day 21.

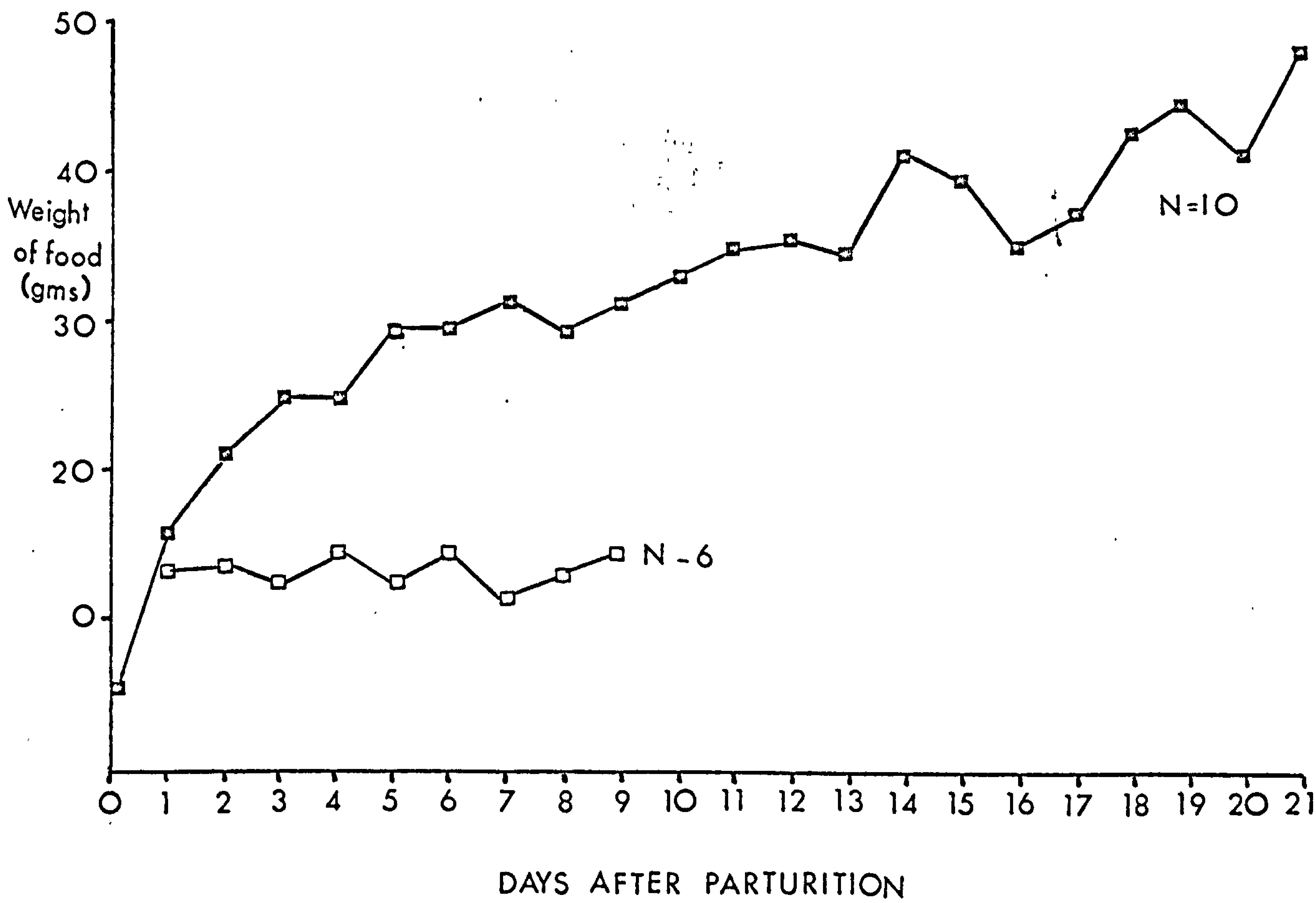


Figure 3: Graph showing mean daily food intake of Day 0 - 21 lactating female rats

- Lactating PVG/C female rats.
- Non-lactating, nulliparous female PVG/C rats.

The three-fold increase in intake was considered to be sufficient to enable pheromone emission to take place. Leon (1974, Fig. 2) presents data which indicates that nulliparous females emitting the pheromone, after receiving prolactin injections, were consuming 20 - 23 gm food per day at the time of testing for pheromone emission.

As a later study by Leidahl and Moltz (1977) indicated that the maternal pheromone could be emitted by concaveated nulliparous female rats, without a concurrent increase in dietary intake, it would seem unlikely that the cause of the failure to replicate lies in this difference between the rats.

What of caecotrophe itself? This "semi-solid light-coloured unformed material" (Leon, 1974), normally eaten by rats, but excreted in much larger quantities by lactating females, apparently contains the maternal pheromone. Was this material being produced satisfactorily by the rats in the Hull colony? Although it was not possible to compare this anal excrement directly with that produced by rats on the other side of the Atlantic, going by the written description, it did not seem that the Hull rats were very different. Their excrement was often too wet to form a typical faecal pellet, it was a much lighter colour (although perhaps due to the fact that it had a higher moisture content, rather than because its composition differed), and could be distinguished from the normal boli by odour - although again, perhaps because it smelt stronger, rather than different from the kind produced by non-lactating rats.

It seemed then, that there was no reason why the rats used in the Hull colony should not produce caecotrophe and the maternal pheromone, and so the next experiment was designed to investigate the more likely possibility that the airflow in the apparatus was inadequate. Bearing in mind that the criterion of goal box entry might be too stringent, observational work on the pups' behaviour, in order to discover whether they spent more time in the vicinity of the maternal odour, rather than elsewhere in the arena, was carried out.

PART II

EXPERIMENT 4: AIRFLOW RATE IN THE APPARATUS

INTRODUCTION

Leon (1974), gave the rate of airflow through his olfactory discrimination apparatus as 55 litres/min. Measurement of the airflow through the apparatus built at Hull indicated that it was only 29 litres/min, and so it was possible that this weaker airflow was failing to convey the pheromone through the apparatus. A stronger motor was fitted to the pump, but unfortunately, when working at maximum capacity, it was still only capable of 49 litres/min; this value still being somewhat below tha American rate. However, three rates of airflow were selected; 0, 25 and 49 litres/min, and were used as the values for the independent variable in this experiment. The rationale was that if attraction to the pheromone, as evidenced by the pups' behaviour, depended upon rate of airflow through the apparatus, then numbers of pups preferring the maternal excrement would emerge as a function of its rate.

As mentioned in Part I of this chapter, the length of time a pup spent in each of the three divisions of the arena would be recorded, so that if pups were attracted towards the source of the maternal pheromone, but merely failed to enter the goal compartment, their preference could be discerned and quantified, even if they did not actually make a "choice" according to Leon and Moltz's criterion.

Purpose

The aim of this experiment was to determine whether the rate of airflow through the olfactory discrimination apparatus was a variable which influenced 18 day old rat pups' choice behaviour. As in Experiment 2, the pups would be given a choice between goal boxes containing excrement obtained from either their own mother or a non-lactating female. In case pups were discriminating the odours present, but approaching the source without entering the goal boxes, the time that each pup spent in the proximity of the excrement, but still in the arena, would be recorded.

Subjects

PVG/C rat pups, tested at 18 days age were used. 132 were obtained from 22 litters born to nulliparous females at 100 ± 10 days, and whose litters had been culled to 6 on the day of birth. Excrement from non-lactating females was obtained from 4 nulliparous females of the same age as the mothers.
Maintenance was as described in Part I of Chapter III; the rats were fed the Oxoid diet.

Apparatus

The olfactory discrimination apparatus, with a more powerful motor driving the airflow pump was used. The motor made a noise of 70 dB. 2 stopwatches were used to time the pups' movements in the 3 parts of the arena.

Metabolic cages were used for excrement collection.

Procedure

Rat pups were deprived of maternal attention for 3 hours before testing began; testing took place over 6 consecutive days, between 9.00 and 17.00 hours daily.

Excrement was collected by means of the metabolic cages described in Part I of Chapter III. Mothers were isolated for 3 hours and non-lactating females for 6 hours.

The standard test procedure with the olfactory discrimination apparatus was used, but for the 3 experimental conditions the rate of airflow through the apparatus varied. The 3 rates were 0, 25 and 49 litres/min. When there was no airflow, the pump was disconnected from the apparatus, but the motor still ran, so that the background noise it made did not alter for this condition.

Although it would have been desirable to run a control group (pups tested in the empty apparatus) for each of the 3 airflow rates, considerations of time and space meant that this was not possible. As it was hoped that the stronger air current would prove successful in eliciting responses from the pups, a control was run only for this group (i.e.with the airflow rate of 49 litres/min.). The control results from Experiment 3 would provide a baseline for the 25 litres/min rate; it was not anticipated that the pups run in the apparatus without airflow would show any marked preference, but if they did, then subsequent investigation would incorporate appropriate controls.

The 3 experimental groups were all tested with the pups given the choice of maternal or non-lactating female rats' excrement placed in the compartments. Samples were of equal weight, between 3 and 5 gm.

In the standard procedure, excrement samples are changed over, and the apparatus washed out after half the litter have been tested. This was done in this experiment, but in addition, two values of the independent variable were used for each litter. This could be achieved very simply by changing the pump motor to the setting necessary for a particular airflow. Counterbalancing was used, so that for a typical litter, run with the two airflow values "A" and "B", the testing conditions would be as follows:

Pup	Side of maternal excrement	Airflow condition.
1	Right hand box	A
2	Right hand box	B
3	Right hand box	A
4	Left hand box	B
5	Left hand box	A
6	Left hand box	B

Five litters were run with the airflow rates of 0 and 25 litres/min; five with 25 and 49 litres/min; and five with 0 and 49 litres/min. An additional litter was tested with a single independent variable value of 49 litres/min., and the six litters comprising the control were run in a single block on their own, with a constant airflow rate of 49 litres/min. This counterbalancing design ensured that side of presentation of maternal excrement and litter effects did not confound the results, but if they were of importance, they were distributed evenly across all conditions.

The time that each pup spent in the 3 divisions of the arena was recorded. Pups were allowed 10 minutes in which to make a choice.

Results

i Goal box entries.

The numbers of pups entering goal boxes containing either maternal or non-lactating female excrement are shown in Table 8.

Table 8

Choice behaviour of 18 day old rat pups when airflow through the olfactory discrimination apparatus altered

RATE OF AIRFLOW (Lit/min)	PUPS' BEHAVIOUR			TOTAL
	Maternal excrement	Nulliparous excrement	Remained in arena	
0	11 (36.6%)	8 (26.6%)	11 (36.6%)	30
25	10 (33.3%)	7 (23.3%)	13 (43.3%)	30
49	10 (27.7%)	9 (25%)	17 (47.2%)	36
	CONTROLS			
	Left hand box	Right hand box	Remained in arena	
49	7 (19.4%)	4 (11.1%)	25 (69.4%)	36

χ = 8.52; df = 6; p = >0.05

Although more pups entered the goal compartments whenever there was any excrement present, χ^2 test carried out on the complete 4 x 3 contingency table was nonsignificant ($\chi^2 = 8.52$; df = 6; p = > 0.05). Thus there was no evidence from the choice behaviour that there was anything other than a slight preference for goal compartments over the arena, with, again, a slight preference for the compartment containing the maternal excrement.

There was no difference between the choices made by the control group in this experiment, and those of Experiment 3 ($\chi^2 = 3.65$; df = 2; p = > 0.05: using averaged goal box entries).

ii Behaviour in the arena

To determine whether pups were attracted towards the source of maternal pheromone without actually entering the goal compartments, it was necessary to eliminate pups which did enter the goal boxes from the analysis. This was because an entry was often made within a minute of the start of timing, and so if these pups' scores were included in the time analysis, they would have indicated only very short periods of time spent in the vicinity of either sample of excrement.

The mean times spent by the pups which failed to enter a goal box in the 3 parts of the arena are given in Table 9. It can be seen that the pups did not show a particular preference for the area of the arena nearest to the source of the maternal odour, and the rate of airflow did not appear to influence their behaviour to an important extent.

Table 9

Mean times spent by pups which did not enter a goal compartment in different parts of the arena

RATE OF AIRFLOW (Lit/min)	TIME IN MINUTES			N
	In front of maternal box	In front of nulliparous box	Away from boxes	
0	2.73	3.2	4.07	11
25	2.13	2.0	5.9	13
49	1.7	1.7	6.6	17
	CONTROLS			
	Left hand box	Right hand box	Away from boxes	
49	2.7	1.6	5.7	25

Conclusion

Taking the two sets of results together, there is no evidence that the rate of airflow influenced the pups' behaviour in any way.

Although with all the groups of pups run, there is a persistent small preference for the maternal excrement, comparison with the control group, and looking at the dichotomy in terms of an a priori 50: 50 division, indicated that the preference was not necessarily anything other than a chance occurrence.

Discussion

The results of the experiment were disappointing, in that not only did the variation in airflow rate fail to influence the pups' choices, but the substantial proportion of pups which failed to enter the goal compartment did not show any signs of preference for the side of the arena nearest to the maternal excrement either.

When the results of this experiment are compared with those obtained in Experiment 2, it can be seen that these more recent results provide even less evidence for the existence of a pheromonal agent than the earlier ones. In Experiment 2, 52% of the pups chose the maternal goal box, whilst in Experiment 4, the average is 32.5%. Before a possible reason for this is put forward, and a final interpretation of the results advanced, the independent variable, airflow rate, will be discussed in a little more detail.

Because the airflow rate still had not reached the strength of that reported by Leon and Moltz, it was not possible, even after the experiment had been carried out to completely rule out the difference in apparatus as the variable preventing the pheromone effect.

Although it seemed unlikely that responsiveness should occur in an all-or-none fashion to a high airflow rate, another possibility was that the female rats used in these experiments released the pheromone in lower concentrations than their American counterparts, and so a swifter airflow was needed. This point is debat able however, and it could be argued equally well that a <u>slower</u> airflow would convey a weaker odour more effectively. The main point was though, that it was still not known whether the attraction failed to appear because the pheromone was failing to reach the pups; because it was not emitted in sufficiently large quantities, or was absent; or because of some inadequacy on the pups' part. Fortunately, soon after Experiment 4 had been run, two pieces of information were obtained which indicated that the reduced airflow rate was quite adequate for the purposes of obtaining approach responses from the pups. First was a letter from Leon (dated 28.5.76; included in Appendix III), in which he states that a pheromone effect can be obtained even when there is no airflow through the apparatus.

Second, was the discovery of some work on the maternal pheromone in mice, carried out by Howard Knoff, an undergraduate student at Keele University. Knoff had also realised that the mothers' presence in the goal compartments presented a confounding variable, and he had modified the olfactory discrimination apparatus so that a stream of air passed over a stimulus animal or excrement before it enters a goal compartment. Knoff's work and findings will be described in a little more detail in Chapter XIII, and at this point it will suffice to say that although he used an airflow rate of only 9 litres/min, some kind of odouriferous attractant was successfully conveyed through the apparatus, as evidenced by the mouse pups' behaviour. On the basis of these two pieces of information, it was decided that the airflow through the apparatus was not likely to be a variable giving rise to the failure to replicate; as there were no other features of the apparatus likely to create difficulties, the apparatus as a whole was discounted as the source of the problem.

To return to the interpretation of the goal box choices made in this experiment, and make a comparison of them with the earlier experimental results. In this, and subsequent discussions of results, a convention will be adopted whereby pups' choices in any particular experiment will always be indicated by three numbers. The first number will be the number of pups entering the maternal compartment, the second, the number entering the non-lactating female's compartment, and the final number, that of the pups remaining in the arena. As most of the statistical comparisons carried out between experimental groups and control groups use the average of the two empty goal box entries for the control group data, averaged figures will normally be given. When control results are not averaged, then the first number will refer to the number of pups entering the left hand goal box, and the second, the right hand goal box.

First, a look at the results obtained from the two control groups run so far. In Experiment 2, the choices made were 10: 9: 17, whilst those of this Experiment were 7: 4: 25. χ^2 test carried out on the two sets of figures (with goal box entries averaged) indicated no difference between them (χ^2 = 3.654, df = 2; p => 0.05). Indeed, to have found a difference between them would have been extremely disquieting, as it would either have indicated that the strength of air current alone, in the empty apparatus, was a variable of importance, or that the pups' responses were changing over a period of time.

When the three experimental groups from Experiment 4 were compared with the control group one by one, no significant differences were obtained, as was also the case when the whole set of results was cast into a 4 x 3 table for analysis. In contrast to this, in

Experiment 2, the results 79: 47: 25, when compared with the control results of 9.5: 9.5: 17, were significantly different ($\chi^2 = 16.46$; df = 2; p = <0.001), and this was taken as providing support for the attractive properties of maternal excrement. It was known then, that in Experiment 2, the pups' behaviour did differ from that recorded when the apparatus was empty, but that in Experiment 4 it didn't.

To determine whether the two sets of experimental animals differed when a direct comparison was made, χ^2 tests were carried out on the results. The 79: 47: 25 obtained in Experiment 2, with an airflow rate of 25 litres/min. was compared first with its equivalent in Experiment 4 (10: 7: 13), and then with the other results of 11: 8: 11, and 19: 9: 17, obtained with airflow rates of 0 and 49 litres/min. respectively. In the same order, χ^2 values obtained were 10.466 (p = <0.01); 6.47 (p = <0.05); and 16.227 (p = <0.001), confirming the impression that the pups' choices in Experiment 4 did differ from the ones made in Experiment 2. Examination of the χ^2 contingency tables made up from these results, following the method suggested by Rosenthal and Rosnow (1975), indicated that in all cases there was one cell which contributed overwhelmingly to the large χ^2 value. This was that of the pups in the experimental groups which had failed to enter goal boxes. Well over a third of them had remained in the arena in all three cases, whereas less than a fifth of them had done this in Experiment 2.

Why should this difference between the two sets of pups have occurred? The only difference in procedure between Experiments 2 and 4 (apart from the airflow rate, which appeared to have no effect), was a change in the method of excrement collection. In Experiment 4, metabolic cages were used for excrement collection, enabling better, although still not complete separation of urine and faecal matter. It seemed that the collection of excrement by this method had impaired the very limited attractant effect which had been obtained earlier. It is known that odour from stressed rats influences the behaviour of conspecifics (Valenta and Rigby, 1968), and it was possible that the mothers, enclosed in the small metabolic cages for 3 hours had secreted a substance whose odour had aversive properties. That the mothers were stressed to a certain extent was known by the fact that they were extremely reluctant to enter the metabolic cages once they had been kept there, and because they never ate any food whilst they were in the cages.

Any secretions which might have been given off by the mother during the 3 hour excrement collection period were not completely aversive, for there was not a complete blocking of the mild attractant effect. In the case of all three experimental groups, more pups chose the mother than the nulliparous females' excrement, although the preference for the maternal excrement was only slight, and did not warrant statistical analysis. Also, whilst more pups remained in the arena than had been the case in Experiment 2, more pups entered goal boxes when the excrement was present than when the apparatus was empty (in the control group trials).

At the end of Experiment 4 then, when the results had been analysed, compared with those obtained from previous experiments, and supplemented by information from other sources, three things were known. One was that the apparatus and airflow rate were adequate for the task in hand. Second, was the conclusion that either the maternal pheromone was absent, or present in reduced quantities; <u>or</u> it was present, but for some reason failing to elicit the appropriate approach response from the pups. Third, the results suggested that the method of excrement collection employed influenced the pups' behaviour, and so should be regarded as a variable of relevance in subsequent experiments.

CHAPTER VII

THE ABILITY OF PVG/C RAT PUPS TO MAKE OLFACTORY DISCRIMINATIONS

INTRODUCTION

After Experiment 4 had been run, it was concluded that the failure to obtain any evidence of a maternal pheromone could not be attributed to the apparatus, but must lie in the pups themselves, or with the mothers. It was essential to establish that the PVG/C rat pups used in these investigations could smell, and that they were capable of making discriminations based on olfactory cues. The experiment reported in this chapter was undertaken in order to determine whether the pups were adequate in these respects.

EXPERIMENT 5: DISCRIMINATION OF MATERNALLY SOILED PAPER BY 12 DAY OLD PVG/C RAT PUPS

Purpose

Jones and Nowell (1974) used an extremely simple technique when they investigated the effects of urine substrates upon mouse behaviour. A mouse was placed in a test cage containing a plain paper floor, half of which had been spotted with urine obtained from another mouse, and half of which was clean. The time which the mouse being tested spent in the two halves of the enclosure was then recorded.

A modified version of this test was used in Experiment 5 to determine whether 12 day rat pups were capable of olfactory discrimination.

A paper sheet which had previously been soiled by the mother was placed in one half of a plain rectangular enclosure, on the floor. If the majority of the pups displayed a preference for either this or the clean side, then this behaviour would provide evidence that olfactory discrimination had occurred. Pups were tested at 12 days as their eyes were not yet open, and so their choice would be based only upon olfactory cues.

As pups would be recorded as being on one side of the box or the other, and there was no third category, the binomial test, with a probability of $P = Q = 0.5$, would be used for analysis of the results. With 48 pups tested, 32 would have to select one side of the box before the Null Hypothesis could be rejected ($p = 0.031$, two-tailed test).

Subjects

48 PVG/C rat pups obtained from 8 litters born to females which had been mated at 100 ± 10 days. All litters had been culled to 6 on Day 0, and pups were tested on Day 12.
The adult rats were maintained in the usual manner, and had been fed the Oxoid diet.

Apparatus

A plain rectangular box, 50 x 36 x 15 cm, constructed of white plexiglass, comprised the test arena. During test sessions a tightly fitting transparent lid was placed over the enclosure.
Two stopwatches were used for timing purposes.

Procedure

The test cage was lined with a single piece of plain white paper, and a wire grid placed over it. A 12 day lactating female was taken from her litter and placed in the box, without food or water, for a period of one hour. She was then removed from the box, and kept in a holding cage until all the pups in her litter had been tested. For the test, the wire grid was removed and the paper sheet taken out of the box. Any solid matter, such as faeces or fur, was shaken off it, and it was then cut in half, across the long side. The test box was thoroughly washed out and lined with a clean paper sheet. On top of this, one half of the soiled paper floor was placed, in such a way that the test box now had a paper floor with a central line down the middle where the soiled sheet ended.

Pups were placed singly in the centre of the test box, and the transparent cover was put into place. The time which the pup spent in each half of the box was recorded, for 5 minutes. If a pup touched the central line where the soiled paper ended, then this time was subtracted from the overall score, but was not allowed in addition to the 5 minutes. Pups tended to settle in one place after a period of exploration, and their final resting position was also recorded.

When a trial ended, the pup was removed and placed with its mother in the holding cage. The apparatus was washed and turned round, so that for the next trial the soiled paper was on the opposite side. The bottom sheet of paper was replaced with a clean sheet. This procedure was repeated for all the pups from any one litter; after a complete litter had been tested, the paper sheets were discarded and a new soiled sheet obtained from the mother of the next litter to be tested. Testing took place over a three day period.

Results

The sides of the test box in which the pups settled, and the length of time they spent in the two halves are given in Table 10.

Table 10

Olfactory discrimination made by 12 day rat pups, using maternally soiled paper as stimulus material

Side of test box	No of pups settling on each side	Mean time per pup in each side
Soiled paper	35	3.35 mins
Clean paper	13	1.47 mins
Total	48	4.82 mins

Thirty-five pups preferring one side of the test arena exceeded the minimum of 32 needed for rejection of the Null Hypothesis. The exact probability of 35 out of 48 pups preferring one side is 0.002 (binomial test, two-tailed, $z = 3.03$).

The pups spent over twice as much time on the side of the box which had the soiled paper floor; on the basis of these results, it was concluded that the 12 day rat pups tested were capable of making olfactory discriminations.

Discussion

First of all, it seemed clear that the 12 day old rat pups could smell. Secondly, it is of interest to look at the results of this experiment within the wider framework of the maternal pheromone investigations. For the first time, there was some evidence of attraction towards an odour derived from the mother. However, whether pups would prefer this to odours derived from non-lactating females was still unresolved. As Leon and Moltz had found that 12 day rat pups were not attracted towards the maternal stimulus in their olfactory discrimination apparatus, such a preference would not be anticipated; at this stage, the behaviour of 12 day rat pups was not investigated any further.

The way that the soiled paper sheet had been obtained meant that it could have picked up a number of different odours, from the faeces and urine, but also from the mother's ventral surface, fur and ano-genital region generally. The pups' preference for this soiled paper need not necessarily be a reflection of the presence of a maternal pheromone in the faeces.

As the pups' eyes were not yet open, their preference could not have been based on visual stimuli. This might have been possible with older pups, as the soiled paper was a little stained by urine in places. However, the soiled paper sheets were not sufficiently covered in urine or excrement over the 1 hour period to become soggy; both sheets of paper in the arena during testing periods were quite dry.

This simple testing technique was in many ways better for obtaining preferences than the more complex olfactory discrimination apparatus.

There was no pump motor noise frightening the pups, and although they were being placed into an open-field situation, the test comprised this alone, rather than an open-field plus exit points.

It was noticed that when pups were removed from the test box after 5 minutes, they were quite cold, a fact which probably helped to bring about their immobility. In this simple apparatus, their loss of body heat is not very important, as their choice of side involved very little movement. Its occurrence means though, that it could affect the results obtained from such young pups tested in the olfactory discrimination apparatus, where after 3 hours separation from the mother, the pups are relatively cold <u>before</u> testing starts, and where they are required to move about half a metre. The implication of this will be discussed in Chapter XIV.

Conclusion

The results of Experiment 5 indicated that the PVG/C rat pups used in the investigations into the maternal pheromone could smell, as they were capable of making discriminations on an olfactory basis. The fact that they did not seem to respond strongly to the maternal anal excrement could no longer be attributed to any deficiency in olfaction on their part. Instead, it might be that they <u>could</u> make the discrimination, but for unknown reasons failed to enter the maternal goal box. This possibility is explored in subsequent experiments.

CHAPTER VIII

PILOT STUDIES ON OLDER PUPS, USING HOME CAGE BEDDING, URINE AND IMMOBILE BODIES AS STIMULUS MATERIAL

INTRODUCTION

In the first four experiments, 17 and 18 day old rat pups were tested in the olfactory discrimination apparatus, using the standard procedure described in Chapter III.

After the pups had been run in the apparatus once, they could not be used again in any of the main experiments, yet it was felt that these post-18 day pups could be used for other work, rather than disposed of immediately. A second testing provides two confounding variables; experience in the apparatus, and age. However, as the first experiment had demonstrated so clearly that what seemed fine in principle did not necessarily work in practice, no harm could be done by trying out new techniques, or making preliminary investigations with pups which had already been tested once. Consequently, three experiments were undertaken during the course of Experiments 2, 3 & 4, with such rat pups. The results from these pilot studies are reported out of chronological order at this point, as they influenced the direction of later experimental work.

Although it was only known after Experiment 5 had taken place that the failure to replicate could be narrowed down to the pups' disinclination, rather than inability to choose the maternal odour, or to failure of emission from the mother, it had been felt from the start that a longer period of pre-test maternal deprivation, i.e. manipulation of motivation levels, might bring better results. In these pilot studies, motivation levels are altered by increasing deprivation from 3, to 4 or 18 hours. At the same time, in Experiment 6 there is another alteration, in that bedding material from the home cage, rather than maternal excrement was used as stimulus material. Stimulus material was different again in Experiment 7, when maternal urine was opposed to male rat urine in the olfactory discrimination apparatus.

As there had been very little indication of any preference on the part of the rat pups for any kind of maternal excrement, in the third pilot study, Experiment 8, intact animals were used as stimulus material. However, to control for auditory cues, the stimulus animals were anaesthetised before being placed in the goal boxes. The rat pups in this experiment were 24 days old when tested.

EXPERIMENT 6: ATTRACTION OF HOME CAGE BEDDING, WITH 4 HOURS OF MATERNAL DEPRIVATION

Introduction

This experiment, in which the period of pre-test maternal deprivation was increased from 3 to 4 hours, took place immediately after the first experiment, in which problems of excrement collection had arisen. It was decided not only to use bedding material from the home cage, rather than excrement, as stimulus material, but also to increase the length of pre-test deprivation.

Purpose

The purpose of Experiment 6 was to determine whether pups deprived of maternal attention for 4 hours would be attracted towards the goal box in the olfactory discrimination apparatus which contained bedding material taken from the home cage, rather than that containing material taken from the cage of a strange non-lactating female.

Subjects

24 PVG/C rat pups aged 20 days from 4 litters were used. They had already been run once in the olfactory discrimination apparatus.

Apparatus

The rat pups were tested in the olfactory discrimination apparatus.

Procedure

Mothers were removed from their cages 4 hours before testing started. Approximately 50 gm of home cage bedding material (torn paper plus faecal matter), which had been unchanged since the week before parturition, were placed on a fine wire grid in the olfactory discrimination apparatus goal boxes. Bedding material from the pups' own home cages was placed in one of the goal boxes, and the same material from the cages of 3 non-lactating females (approximately 18 gm from each) was placed in the other.

The standard test procedure, using the olfactory discrimination apparatus was followed; all the testing took place on one day.

Results

All the pups made a choice: 14 entered the compartment containing the home cage bedding, and 10 entered the goal box which contained the bedding from the non-lactating female.

The probability of such a division into two, when the binomial test was applied to the results, is 0.542 ($P = Q = \frac{1}{2}$; two-tailed probability).

Thus there is no evidence that the pups displayed any preference for their own home cage bedding.

Discussion

A slightly longer period of maternal deprivation and a larger, familiar amount of material in the goal box still failed to elicit a preference for the maternally derived stimulus. Home-cage bedding would provide what has been called a "medley of aroma". It contained faecal matter, dried urine, and odours derived from direct contact with the mothers' bodies; even so, there was still no sign of any attractant effect.

It was interesting that the 20 day old pups all chose a compartment. As they had already been run in the olfactory discrimination apparatus, their familiarity with the test procedure might have meant that they were less stressed when tested, and so this influenced their behaviour. Or their readiness to enter goal boxes might have been a function of age alone. A third explanation for the change in behaviour is that the slightly longer period of maternal deprivation resulted in altered motivation levels.

EXPERIMENT 7: PUP PREFERENCES FOR URINE

Introduction

In the very first paper on the movement of pups towards a maternal odour, Nyakas and Endröczi (1970) reported that pups were attracted towards the mother's urine. In 1971, Leon and Moltz found that 83% of a group of 16 day old pups entered a goal box in the olfactory discrimination apparatus which had been soiled by the mother, and which presumably contained both her urine and faeces. However, in his monograph "Maternal Pheromone", Leon states that only 33% of a group of 16 day pups entered a compartment containing maternal urine, when opposed to an empty compartment (Leon, 1975, Experiment 1C). It appears that there is a certain amount of confusion over the attractant properties of urine.

Purpose

The aim of this experiment was to see whether 20 day old rat pups were attracted towards maternal urine when this was opposed to urine obtained from male rats.

Subjects

30 PVG/C rat pups, aged 20 days, from 5 litters which had already been run in the olfactory discrimination apparatus were used. Maintenance was normal, and the adult rats had been fed the Oxoid diet. Urine was obtained from 2 male rats, aged 145 $\pm$ 10 days.

Apparatus

The olfactory discrimination apparatus was used for testing the pups. Urine samples were collected in the metabolic cages described in Chapter 3.

Procedure

The rat pups were deprived of maternal attention for 18 hours before testing sessions, which took place over a 3 day period.

Urine for the goal compartments was obtained by placing the mothers and male rats in individual metabolic cages for about 3 hours. The urine which collected below the cage was poured onto cotton wool, and this was then placed in the goal compartments. The standard test procedure was then followed.

Results

Of the 30 rat pups tested, 18 made a choice by entering one of the two goal compartments: 10 chose the mother's urine, and 8 chose the male rats' urine.

A χ^2 test was carried out on the results, in which 10: 8: 12 was compared with the results of 10: 9: 17 obtained from the control group of Experiment 3. It was found that there was no significant difference ($\chi^2 = 0.373$; df = 2; p = >0.05). This suggests that the pups were behaving in the same way that they did when the apparatus was empty.

Discussion

Although there is some disagreement in the published literature as to whether young rat pups will approach maternal urine, it was surprising that the young tested in this experiment did not prefer it when it was contrasted with urine obtained from male rats.

Nyakas and Endröczi (1970) had observed that if live animals were placed in the goal boxes, the mother was approached in preference to a male, and so a discrimination appeared to be taking place. Unfortunately, this experiment was subject to the same confounding variables as much of the work reported by Leon and Moltz, and so the discrimination may not have been due to the operation of olfactory cues. Another point is that the pups tested by Nyakas and Endröczi were only aged 10 days; their attraction to urine samples at this age does not necessarily mean that they will be attracted at a later age. The age at which Leon and Moltz tested rat pups was 16 days, and this difference rules out a direct comparison of results.

At the time this experiment was conducted, it was not known that the method of collecting excrement from metabolic cages might be an important variable. In retrospect, and in view of the later findings from Experiment 4, in which the importance of collection method emerged, these results do not necessarily indicate that there is no attractant present in maternal urine. Its effect might have been attenuated by other secretions produced from a stressed animal.

A problem of interpretation might have arisen if the pups had preferred the maternal urine. This could have been indicative of avoidance of male rat urine, rather than approach to a maternal odour.

EXPERIMENT 8: IMMOBILE BODIES AS STIMULUS MATERIAL

Introduction

Rather than use mobile animals in the goal boxes, as Leon and Moltz did in many experiments, it was possible to obtain the same olfactory cues, but without confounding variables, by using anaesthetised, immobile animals in their place. Immobility could be induced speedily, and with little stress for the animal, by use of Immobilon, a drug manufactured by Messrs. Reckitt and Coleman, Hull. Details of Immobilon are given in Appendix III.

Purpose

This experiment was a pilot study in which a technique for anaesthetising the stimulus animals before placing them in the goal boxes would be tried for the first time. Rat pups would be tested to see whether they displayed a preference for their mother's body, or that of a non-lactating female. The pups would be deprived of maternal attention for 18 hours before testing took place.

Subjects

36 rat pups, aged 24 days, taken from 6 litters which had been already tested in the olfactory discrimination apparatus were used. Two non-lactating females of the same strain, PVG/C, and of the same age as the pups' mothers were also used.

Maintenance was as described in Part I of Chapter III; the adult rats had been fed the Oxoid diet.

Apparatus

The olfactory discrimination apparatus (airflow 29 litres/min) was used for testing the pups.

Procedure

Pups were separated from their mothers the night before testing was to take place, and they underwent 18 hours maternal deprivation.

A few minutes before a test session began, the mother was taken from the holding cage, and given an intramuscular injection of Immobilon (1 ml per kg body weight) sufficient to keep her unconscious for at least an hour. One of the non-lactating females was also given the same drug treatment. The two female rats were then placed in the goal compartments of the apparatus so that they were both lying on their sides, ventral surfaces towards the centre, heads nearest the wall furthest from the arena. Pups were always tested with their own mother.

The standard test procedure with the olfactory discrimination apparatus was followed, with the stimulus animals reversed, washing out, etc., every third trial. Testing took place over three days.

Results

The following responses were recorded:

Pups which entered the mother's box........................	12
Pups which entered the non-lactating female's box..........	22
Pups which remained in the arena...........................	2
Total	36

χ^2 test carried out on these results and the 18 day control group from Experiment 3, was significant at the 0.001 level (χ^2 = 17.09; df = 2). The actual comparison numbers used were 12: 22: 2, against 9.5: 9.5: 17, the latter being the averaged control results.

Discussion

All but two of the pups preferred to enter a goal box, rather than remain in the arena. At first sight, the fact that the majority of pups chose to enter the non-lactating female's compartment seemed bizarre; however, examination of the χ^2 contingency table, and the "expected"frequencies, indicated that most of the χ^2 value lay in the number of pups which failed to enter a compartment, rather than in the other four cells. The binomial test carried out on the thirty-four pups which did make a choice gave a nonsignificant result (p = 0.124), indicating that the preference for the non-lactating female was no more than might have been obtained by chance.

At 24 days age, these pups were almost weaned, and, additionally, they were already familiar with the olfactory discrimination apparatus. It could have been that they were not motivated to obtain food from their mother, even after 18 hours deprivation, but being less fearful of the test procedure, they exhibited more exploratory behaviour and entered the goal boxes as one consequence of this. Another possibility, briefly mentioned in Experiment 6, was that older pups tend to enter goal compartments regardless of familiarity with the test procedure or apparatus - later, the results of Experiment 10 indicated that this was the case.

Summary of Experiments 6, 7 and 8

It is difficult to interpret the results from these experiments, as there were several confounding variables present. However, the work did give pointers towards variables which might be worth examining, and in Experiment 8, the technique using the drug Immobilon had proved successful, and did not appear to have any drawbacks.

In the light of the results from these experiments, it was decided to carry out an experiment in which naive 18 day rat pups were deprived of maternal attention for 18 hour periods befor being tested with faecal matter or immobile bodies as stimulus material in the goal boxes in subsequent experiments. Also, the behaviour of 24 day old rat pups in the olfactory discrimination apparatus, under standard conditions, would be examined, in order to determine whether it differed from that shown by younger animals.

Both experiments, which are reported as Experiments 9 and 10, would be run with their own control groups.

CHAPTER IX

STUDIES OF PRE-TEST MATERNAL DEPRIVATION AND OLDER PUPS

INTRODUCTION

Two sets of experimental work will be covered in this chapter. The pilot studies which were reported as Experiments 6, 7 and 8 in the previous chapter indicated that the length of time which pups were deprived of maternal attention - or what might be called motivation - could have some bearing on whether the pups were attracted towards the maternal stimulus.

In Part I, an experiment in which pups were isolated from their mothers for 18 hours before being given the choice of different types of faecal material, or two anaesthetised animals in the goal boxes is described.

Pups aged 24 days which had been run in the pilot studies made a high proportion of goal box entries, and so a set of comparisons in which the age of the pup is the independent variable was undertaken, and is reported in Part II of this chapter, as Experiment 10.

Part III comprises a summary of the conclusions reached after Experiments 9 and 10 had been conducted.

PART I

EXPERIMENT 9: PRE-TEST MATERNAL DEPRIVATION OF 18 HOURS

Introduction

In Experiment 6, when the pre-test period of maternal deprivation had been slightly increased to 4 hours, there was still no evidence of any greater tendency for the pups to approach the stimulus material. A further increase to 18 hours had not produced approach responses or discrimination when urine samples were used, but when 24 day pups were tested after the same period of deprivation, in Experiment 8, with immobile bodies, a much greater proportion chose to enter the goal boxes. However, it was not known whether this propensity was due to the nature of the stimulus material, or a function of pup age.

In this experiment, three sets of 18 day old pups were run, after having undergone 18 hours maternal deprivation. Pups of this age are capable of surviving in the complete absence of the mother; their body temperatures remained high, and they were very mobile when placed in the test apparatus. One set of pups was to be tested with faecal matter providing the stimulus material, another with anaesthetised animals in the goal boxes, and the third group comprised the control, tested in the empty apparatus.

Purpose

To determine whether 18 day old rat pups would be attracted to maternally derived stimuli if they had been deprived of maternal attention for 18 hours before testing took place.

Subjects

108 PVG/C rat pups, aged 18 days, derived from 18 litters born to female rats which had been mated at 100 ± 10 days, were used. Faecal matter was obtained from 6 nulliparous females of the same age as the mothers (140 ± 10 days when testing took place), and three of these females were anaesthetised for use in the second set of comparisons.

Maintenance was as described in Part I of Chapter III; the adult rats were fed the Oxoid diet.

Apparatus

The olfactory discrimination apparatus was used for testing the rat pups, the airflow rate was 29 litres/min.

Excrement was collected by means of the metabolic cages.

Procedure

Mothers were removed from the home cages 18 hours before their particular litters were to be tested. The 18 litters were divided into three groups of six, and the comparisons to be made were:-

Group 1	Anal excrement: mother's versus nulliparous females'.
Group 2	Immobile bodies: mother versus nulliparous female.
Group 3 (Control)	Empty goal boxes.

The standard test procedure was followed, and testing took place over a period of 10 days.

Results

The results of Experiment 9 are given in Table 11.

Table 11

Choice behaviour of 18 day old rat pups which have undergone 18 hours pre-test maternal deprivation

GROUP	PUPS BEHAVIOUR			N	p
	Entered maternal goal box	Entered non-maternal goal box	Remained in arena		
1 (excrement)	10	11	15	36	n.s.
2 (bodies)	15	16	5	36	0.001
	CONTROL				
	left hand box	Right hand box	Remained in arena		
3 (empty)	8	6	22	36	-

The significance levels refer to comparisons carried out between each set of data and the control group, using χ^2 test, and the latter having the two goal box entries averaged to 7.

There was no preference for the maternal excrement (χ^2 = 2.74;

df = 2), but when immobile bodies were placed in the goal boxes, more of the rat pups entered them than when they were empty (χ^2 = 17.134; df = 2). Despite this higher number of goal box entries, there was no evidence of any preference for the mother to the nulliparous female.

Discussion

Before the effect of the increased deprivation period upon the pups' behaviour is assessed, the control group results will be examined. The averaged results of 7: 7: 22 were compared with those obtained from the control group run in Experiment 2 (9.5: 9.5: 17, averaged), and were indistinguishable (χ^2 = 1.397; df = 2). Thus deprivation levels alone did not influence the pups' behaviour in the testing situation, and it was known that approximately one third could be expected to enter the goal compartments unless the contents exerted an influence upon them.

The results obtained from the six litters tested in Group 1 indicated that the excrement samples were not acting as attractants, as evidenced by the fact that these results are indistinguishable from those of the control group. As the pups must have been hungry after 18 hours without their mother (and this was shown by their immediate and keen suckling when they were reunited), it was concluded that neither the maternal, nor the non-lactating females' excrement contained an attractant whose effect depended upon a sufficient level of motivation.

Much more promising results were obtained from the second group of pups, which were presented with the choice of two immobile bodies in the goal compartments. Although they failed to discriminate the mother from the other female, the fact that almost all of them left the arena suggested that some kind of attractant was present. As the stimulus animals were both completely motionless, this attractant must have been olfactory in nature. Because this was the first proper experiment in which immobile bodies were used as stimulus material, it was not known whether the pups' behaviour was dependent upon an 18 hour deprivation period, or whether they would respond in the same way to immobile bodies after 3 hours deprivation. This question was not examined subsequently by work in which the olfactory discrimination apparatus was used, but by a different method, reported as Experiments 16 and 17.

Finally, a comparison of the results obtained from the first group was made with the data obtained from Experiments 2 and 4, where rats had been given the choice of excrement samples. It will be recalled that some difference between the results of these two earlier experiments had been evident, and that this was attributed to the method of excrement collection. The results of Group 1 of this experiment, (10: 11: 15), were indistinguishable from the 10: 7: 13 obtained in Experiment 4, when the airflow had been 25 litres/min., ($\chi^2 = 0.489$; df = 2; $p = > 0.05$). Both had utilised metabolic cages as a method of excrement collection, and similarly, the more recent results differed significantly from those of Experiment 2 ($\chi^2 = 18.45$; df = 2, $p = < 0.001$), where there had been a suggestion of attraction to the maternal excrement when it had been collected by means of small holding cages.

Conclusion

There was still no evidence of the presence of a substance with attractant properties in maternal excrement, as even after prolonged deprivation the pups failed to apprcach the mother's excreta.

Although the young were influenced by olfactory cues, and showed approach tendencies towards immobile bodies in the goal boxes, it was not known whether this was a function of the prolonged maternal deprivation, or due to attractive olfactory properties of the bodies, which might exert their influence upon the pups even without pre-test deprivation.

PART II

EXPERIMENT 10: PUPS TESTED AT 24 DAYS AGE

Introduction

The American researchers note several times that maternal pheromone is emitted by the mother between 14 and 27 days postpartum (Leon and Moltz, 1972; Moltz, Leidahl and Rowland, 1974; Leidahl and Moltz, 1975; Leon, 1975). This statement seems to be based on the results of a single experiment reported in 1972 by Leon and Moltz, (Leon and Moltz, 1972). Some peculiarities of the American work undertaken on the times of emission and response are evident, and these will be discussed in Chapter XIV.

As there was little evidence that pups were responding to a pheromone at 17 and 18 days age, and encouraged by the results from Experiments 6 and 8, where many of the older pups had entered goal boxes rather than remain in the arena, it was considered feasible that the rats used in the Hull laboratory were emitting a pheromone, but at a later age than the American Wistar rats. Consequently, another set of tests was undertaken, using the olfactory discrimination apparatus and standard test procedure, but with 24 day old pups.

Once more, three groups were established. This time, they were a control group, a group given the choice of anal excrement, and one presented with home cage bedding material in the goal compartments.

In the pilot study reported as Experiment 8, 24 day old pups had been tested with immobile bodies present in the goal boxes. At the time of the study, older animals had not been tested before, but as these animals were being tested for the second time, it was not known whether their preference for the goal boxes could be ascribed to their maturity, or the fact that they were familiar with the apparatus. The setting up of a control group in this experiment meant that the results obtained from the pilot study, with its confounded variables, could be evaluated.

Purpose

The aim of Experiment 10 was to discover whether rat pups aged 24 days were attracted towards material derived from their mother or home cage, in preference to that from a non-lactating female.

Subjects

108 PVG/C rat pups, aged 24 days, born to 18 female rats which had been mated at 95 ± 10 days were used.

Faecal matter, and home cage bedding was obtained from 5 nulliparous females of the same age as the mothers used in the experiment, which were housed singly in cages identical to the ones occupied by the mothers and litters.

Maintenance was as described in Part I of Chapter III; the adult rats were fed the Oxoid diet.

Apparatus

The olfactory discrimination apparatus, with an airflow rate of 29 litres/min was used for testing the rat pups.
Excrement was collected from the adult animals by means of metabolic cages.

Procedure

As in the previous experiment, the 18 litters were divided up into three groups of six. The test material for each group is given below.

Group 1	Anal excrement: maternal versus nulliparous female.
Group 2	Bedding material: home cage versus that from nulliparous females.
Group 3	Empty goal boxes.

When bedding [illegible] to be used as stimulus material, approximately 50 gm was taken from the nesting area of the home cage, either of a particular litter, or from the cages of several nulliparous females. Excrement was collected by isolating the donor animals in the metabolic cages for 3 hours.

The standard test procedure was followed, after pups had undergone 18 hours of pre-test maternal deprivation.

Results

Table 12 shows the results obtained from Experiment 10.

Table 12

Results obtained from 24 day old rat pups which had undergone 18 hours pre-test maternal deprivation

GROUP	PUPS' BEHAVIOUR			N	p
	Entered maternal goal box	Entered non-maternal goal box	Remained in arena		
1 (excrement)	11	22	3	36	n.s.
2 (bedding)	23	9	4	36	n.s.
	CONTROL				
	Left hand box	Right hand box	Remained in arena		
3 (empty)	14	18	4	36	-

χ^2 tests were carried out between the two sets of results and the control group results, but the values obtained were nonsignificant ($\chi^2 = 2.012$; df = 2 (group 1); $\chi^2 = 3.216$; df = 2 (group 2) with averaged control figures).

Thus the results indicated that pups aged 24 days were not attracted towards anal excrement or home cage bedding, when these kinds of stimulus material were placed in the goal compartments of the olfactory discrimination apparatus.

Taking the numbers of pups which entered goal boxes only, it was possible to determine whether the pups demonstrated a <u>preference</u> for the maternal stimulus material, by comparing them with the 50:50 division which would comprise the Null Hypothesis. For the pups given the choice of excrement in the goal boxes, the division into 11 which preferred the maternal excrement and 22 the nulliparous females' excrement was not significantly different ($z = -1.74$; $p = 0.08$, binomial test, two-tailed probability). There was an indication of preference for the home cage bedding shown by the pups in Group 3, where the division into 23:9 meant that the Null Hypothesis could be rejected ($z = -2.298$; $p = 0.022$; binomial test, two-tailed probability).

Discussion

Whilst the results are dissimilar to those reported by Leon and Moltz for 24 day old rat pups, and do not support the notion that maternal excrement contains a pheromone with attractant properties, they do suggest that 24 day old rat pups prefer a stimulus with a familiar odour.

The results show (by comparing the control group from this experiment with that run in Experiment 9) that 24 day old pups are more likely to enter goal boxes than 18 day pups given the same period of pre-test maternal deprivation. There is a significant difference between the two groups (comparison data 7: 7: 22, and 16: 16: 4, $\chi^2 = 19.5$; df = 2; p = 0.001), indicating that an increased number of entries into the goal boxes could be a function of age alone, and not necessarily due to experience in the apparatus, or qualities of the stimulus material, as had been speculated after Experiment 8 had been run.

It is not known why there should be such a strong reversal of goal box entries between the experimental groups run in this experiment. Even though the metabolic cages had been used for excrement collection, there was no decline in the actual number of goal box entries made when the results from the group tested with excrement samples were compared with those from the home cage bedding or control animals. Whether the isolated mothers were stressed more than the nulliparous females, and secreted a substance with aversive properties remains a matter of speculation.

It is known that maternal female rats will respond to ultrasonic calling from young pups (Smith, 1975), and as the metabolic cages were in the colony room where all the experimental animals were housed, including the young awaiting testing, it is conceivable that the mothers were agitated by the sounds made by the young rats in the room. However, it is something of a mystery that this should happen in this particular experiment, and not, for instance, in Experiment 4, when metabolic cages had also been used. It should be remembered though, that in the case of both Group 2 in this experiment, and Experiment 8, the numbers of pups entering the non-maternal goal boxes were not significantly different from those which might have been expected to do so on a chance basis.

If one thinks about how 24 day old rat pups might behave in this testing situation, and if one had never heard of the maternal pheromone, then the results are probably what would have been predicted. Rat pups normally start to eat the adult diet at about 14 days age, and those tested in this experiment, 10 days later, were virtually weaned. Indeed, the age at which laboratory rats are separated from their mothers in many animal colonies (including the one at Hull) is 21 days. So, for older pups, there does not seem to be any reason why they should seek out their mother's excrement, or any stimulus associated with her, as she no longer provides a source of food. If the test procedure constitutes a stressful event, then pups might enter goal boxes which contain material with some kind of familiar odour, rather than remain in the arena. The goal boxes are dark, and for this reason also, present a more attractive alternative than the brightly lit arena.

In retrospect then, it seems reasonable that there should be no evidence of attraction to a pheromone, or maternally derived odour for pups of 24 days age, despite published reports to the contrary.

Conclusion

Rat pups of 24 days age have a greater tendency to enter the goal compartments of the olfactory discrimination apparatus than do 18 day old pups. Although they prefer the goal boxes to the arena, they do not choose either excrement or home cage bedding when it is opposed to the same material which has been derived from non-lactating female rats. On the other hand, there is an indication that home cage bedding is relatively more attractive than strange bedding.

The lack of motivation for seeking out the maternally derived stimulus may be due to the fact that at the age of 24 days, the young are weaned and no longer dependent upon the mother for survival.

It is not known which feature of the pups' "age" brings about the greater tendency to enter goal boxes. Perhaps it is a change in exploratory behaviour which accompanies the weaning process.

PART III

SUMMARY OF CONCLUSIONS FOR EXPERIMENTS 9 AND 10

The two experiments reported in this chapter, conducted after pilot studies had indicated that well-motivated or older pups might be attracted towards some kind of maternal odour - if not actually the maternal pheromone, proved disappointing.

Even after 18 hours deprivation, and when known to be hungry, the 18 day old pups did not seek out their mother's body or her excrement, but remained in the arena of the olfactory discrimination apparatus. In contrast to this, the 24 day pups preferred the goal compartments to the arena, but failed to show any preference for the maternal excrement. However, when it was considered that these pups were weaned, this lack of approach did not seem too remarkable. The older pups did show a slight preference for the familiar home cage bedding though.

After Experiments 9 and 10 had been run, the conclusion that the mothers used in those studies were not emitting the maternal pheromone seemed almost inescapable. However, one last possibility, still considering the lack of orientation towards the pheromone as being due to some inadequacy on the pups' part remained. This was that the pups could discriminate the maternal odour, but failed to move towards its source because they were experiencing considerable stress in the test procedure. An experiment designed to investigate this hypothesis is reported in the next chapter.

CHAPTER X

THE EFFECT OF HANDLING UPON THE RAT PUPS' BEHAVIOUR

INTRODUCTION

Three broad hypotheses were advanced in Chapter VI in an attempt to explain why there was no evidence of the maternal pheromone obtained in the rats tested in the Hull laboratory. The first, in which deficiencies in the olfactory discrimination apparatus were suspected, had been eliminated in Experiment 4, when airflow rate was manipulated as the independent variable. From these results, and information obtained from other sources, it was known that the apparatus itself was adequate for the task in hand.

Secondly, inadequacy on the pups' part was advanced. Although it was known after Experiment 5 had been conducted that rat pups from the Hull colony could smell, it seemed possible that they were being tested at too young an age. Whilst pilot studies had indicated that older pups made more goal box entries when tested in the olfactory discrimination apparatus, the results from Experiment 10 showed that this was purely an age-related change, and that these older pups did not approach the maternal stimuli in preference to the non-maternal goal box contents either.

Increasing the pups' motivation, by depriving them of maternal attention for 18 hours before testing did not seem to be a necessary condition for the appearance of the pheromone's powers of attraction. As the pups were consistently failing to display any signs of attraction towards an odour (and it is only through the pups' behaviour that such an attractant's existence can be inferred), it was beginning to look as if the mothers were not emitting the pheromone. Before this third hypothesis was investigated, one further aspect of the pups' behaviour was explored.

It had been noticed that many pups responded to being placed in the apparatus by showing signs of stress - notably freezing or darting behaviour and defaecation. Stress is known to inhibit exploratory behaviour (Montgomery and Monkman, 1955; Stevens and Koster, 1972), and if the open field part of the test constituted a stressful event, then this itself might prevent the pups from responding by moving towards an attractant. If pups were familiarised with the olfactory discrimination apparatus and test procedure <u>before</u> they were confronted with a discrimination task, then instead of reacting to the open field with fear, they might be more likely to respond to stimulus features within the test situation, rather than make a gross response to the test itself.

Experiment 11 was undertaken in an effort to determine whether it was the pups' reaction to stress which prevented orientation towards the pheromone.

EXPERIMENT 11: TESTING OF 17 DAY OLD RAT PUPS HANDLED AND FAMILIARISED WITH THE OLFACTORY DISCRIMINATION APPARATUS.

Introduction

Placing rat pups into a piece of apparatus has two major components regardless of what is expected of the animals once they are actually in it. The first aspect is the removal of the rat pup from the home cage. Often the pup will have been abruptly woken before coming into contact with a human hand, perhaps for the very first time. The second is that the pup is placed in a strange environment, and usually a rather large, noisy and bright one by comparison with the home cage, which has been the only environment so far experienced by the young animal. Any reactions to these novel experiences must affect the pups' behaviour in the test situation, and this experiment was designed to assess their influence upon the pups.

The handling of rat pups, its consequence both directly on them, or as mediated by the mother, is a much investigated and controversial area of interest (Daly, 1973; Lee, 1975). For the purpose of this experiment it was only necessary to consider that handling, as part of the test procedure, might be a factor which was in some way affecting the dependent variable of entry into goal compartments. If rat pups were used to being picked up and removed from the home cage, then the test procedure would constitute a less stressful event, and the pups might respond to an attractant present in the environment, rather than freezing or darting.

The similarity of the olfactory discrimination apparatus to the open-field test has already been commented upon in Chapter V. Again, a great deal of work has been undertaken by psychologists in an effort to understand this seemingly simple, yet, it would transpire, very complex situation (Denenberg, 1969; Ivinskis, 1966, 1968, 1970; Russell, 1972). Often used as a measure of emotionality, the open-field test is undoubtedly a stressful experience to naive animals. Although the olfactory discrimination apparatus does not have bright lights immediately above the arena, or loud white noise played into it, it is certainly well lit, and the motor driving the airflow pump provides a constant background noise of 65 dB. If pups were familiarised with the arena and the stimulation it provides before being given a discrimination test in it, then they might be less traumatised by the proceedings on Day 17 or 18.

To see whether animals which were given this opportunity to explore the arena differed from pups which had received some form of handling treatment only, a comparison group of animals was established. Whilst the pups from one group were allowed time in the apparatus, pups from a matched group would be stroked gently for the same period. Stroking is the handling treatment designated "Type II" by Bovard (1958) in one of the many papers on the consequences of infantile stimulation.

Unlike the rat pups tested in the olfactory discrimination apparatus so far, these two groups would be subject to daily disturbance, and so it was necessary to establish a control group which would be tested under identical conditions, using the standard procedure, but which had also been disturbed daily, and which could be compared with a group of pups which were tested after having been reared totally undisturbed, as in previous experiments. Considerations of time and space meant that each group in this experiment could not have its own control of pups tested in the empty apparatus. It was considered sufficient to compare the results obtained with the undisturbed group with the results obtained from Experiment 4; provided these base-line data did not differ, then Experiment 3 results could be used for control comparison data.

Since the divisions on the arena floor had first been devised in Experiment 4, pups' behaviour had always been quantified by two means, in addition to the number of goal compartments entered. During the 10 minute test period, the amount of time spent by the pup in all three divisions of the arena was recorded (see Figure 2 for plan of the arena). The length of time spent in the triangular part, away from the goal boxes, was discarded, leaving two scores for the times spent in the vicinity of the two goal box doors. Secondly, an ambulation score was obtained by counting the number of times each pup crossed a dividing line on the arena floor. These measures were not included in analyses of the results obtained in Experiments 9 and 10, as they did not contribute anything more towards interpretation of the pups' behaviour than could be gleaned from the number of entries into the goal compartments. In this experiment, it was anticipated that the handling treatment might affect not only the pups' choice behaviour, but also their movement in the arena.

Excrement samples only were to be used as stimulus material in the goal boxes.

Purpose

The purpose of Experiment 11 was to determine the effect upon behaviour of some kind of daily disturbance to the pups between Day 2 and Day 17 when they were tested in the olfactory discrimination apparatus.

One group of pups was to be handled, another to be placed in the test apparatus, and a third to be merely disturbed by the removal of the mother from the home cage. The results obtained from these animals could be compared with ones which were not disturbed before testing (the fourth experimental group), and those obtained from the earlier control group, Experiment 3.

Three dependent variables would be measured; the number of goal box entries made by the pups, time spent in each of the three parts of the arena, and an ambulation score.

Generally, it was hoped that the group of pups familiarised with the test procedure would be attracted towards the maternal pheromone, as reflected by either higher maternal excrement goal box entries, or time spent in the vicinity of the goal box door nearest the maternal odour. The pups subject to handling treatment only would come between them and the other two groups on both scores, with the undisturbed group showing similar results to those obtained from the equivalent group in Experiment 4 (the group with the 25 litres/min. airflow rate). Specific predictions about the effects of handling etc., on ambulation scores were not made, although it was anticipated that differences between the four groups would be evident.

Subjects

179 PVG/C rat pups born to 18 female rats mated at 100 $\pm$ 10 days were used. Litters were culled to 6 at birth, but one pup died on the fifth day after the experimental treatment had started, and so one litter comprised 5 pups. Testing took place on Day 17.

Faecal matter was obtained from 6 nulliparous females of the same age as the mothers used in the experiment; this was obtained from all the adult animals by means of the metabolic cages.

Routine maintenance was as decribed in Part I of Chapter III; the adult animals were fed the Oxoid diet.

Apparatus

The olfactory discrimination apparatus, with an airflow rate of 29 litres/min was used for testing the rat pups.
Excrement was collected by means of the metabolic cages.

Procedure

i The pre-test procedure

Before testing the pups on Day 17, the litters were randomly divided into four groups of six. The treatment given to the four groups was as follows:

Group 1: Apparatus familiarisation. Pups were placed in the open-field area of the olfactory discrimination apparatus and left there for one minute.

Group 2: Handled group. Each pup was stroked gently down its back whilst being held in the Experimenter's hand for one minute.

Group 3: Disturbed group: The mother was taken from her litter and placed in a holding cage for 8 - 10 minutes.

Group 4: Litters were undisturbed from culling to testing.

As litters were born they were allocated to one of the four groups and given an identifying number. The litters in the three groups which were to be disturbed in some way were matched, and when the experimental treatments started, they were carried out on the three simultaneously, as will be described.

Experimental treatment from Day 2 - 16

Three matched litters in their cages, one from each of the groups, were placed on a table near the test apparatus.

The mothers from each cage were taken out, and placed in small holding cages immediately below their own home cage. A wire grid divider was placed in the home cage, with all the pups in the litter placed on the side nearest to the Experimenter.

A pup from Group 1 cage was removed and placed into the olfactory discrimination apparatus; as the cover was put into place, a stopwatch was started.

A pup was taken from Group 2 cage, transferred to the Experimenter's left hand, and stroked down the back with the right index finger for 50 secs.

When the stopwatch read 1 minute, the Experimenter, still holding the Group 2 pup in her left hand, removed the Group 1 pup from the apparatus and returned it to its home cage, pla cing it on the far side of the dividing grid. The Group 2 pup was then returned to the far side of the grid in its home cage.

This treatment was repeated with different pups until all the pups were on the far side of the dividing grid. The three mothers were then returned to their respective litters, and the whole procedure repeated on the remaining fifteen litters in Groups 1, 2 and 3.

This treatment was carried out daily, between noon and 18.00 hrs., from Day 2 to Day 16 inclusive.

As stated earlier, pups from the litters in Group 4 were left undisturbed between culling and testing.

ii The test procedure

On Day 17, all pups were tested in the olfactory discrimination apparatus, using the standard test procedure. Rate of airflow through the apparatus was 29 litres/min. Pups were given a choice of excrement samples, these having been obtained from the pups' own mothers, and any three of the six nulliparous females. These animals were isolated in the metabolic cages three hours before testing took place.

Results

Entries into the goal compartments made by the four groups of animals are shown in Table 13, along with the control results from Experiment 3.

Preferences for the sides of the arena are given in Table 14, and ambulation scores in Table 15.

i Goal box entries

Table 13

Results obtained from pups tested on Day 17 after handling treatment

TREATMENT	PUPS' BEHAVIOUR			N	p
	Entered maternal goal box	Entered non-maternal box	Remained in arena		
GROUP 1 (Familiarised with apparatus)	5 (13.88%)	6 (16.66%)	25 (69.44%)	36	ns
GROUP 2 (Handled)	5 (13.88%)	3 (8.33%)	28 (77.7%)	36	0.05
GROUP 3 (Disturbed)	8 (22.85%)	4 (11.42%)	23 (65.71%)	35	ns
GROUP 4 (Standard)	17 (23.61%)	13 (18.05%)	42 (58.33%)	72	ns
	Entered left-hand box	Entered right-hand box			
CONTROL (Experiment 3)	10. (27.77%)	9 (25%)	17 (47.22%)	36	-

Probability values obtained when each groups results were compared with the control group preferences, using χ^2 test, are indicated in the final column.

Although several months had elapsed since the experiment on airflow rates had been carried out, the results from the undisturbed pups (Group 4), did not differ from those obtained by the group tested with an airflow rate of 25 lit/min in Experiment 4. The comparison figures were 17: 13: 42 (Group 4, Experiment 11), and 10: 7: 13 from Experiment 4. $\chi^2 = 2.9$; df = 2; p => 0.05.

None of the groups indicated any preference for the maternal excrement; with the exception of the handled animals, their choices did not differ significantly from those which were made in an empty apparatus.

The handled animals (Group 2), differed from the control pups. Not, unfortunately, by displaying preferences for the maternal excrement, but because almost 80% of them remained in the arena, in contrast to less than 50% of the control group.

The conclusions drawn from the pup entries into goal boxes will be considered when the other data have been examined. The main effect of handling, however, seemed to be to cause the pups <u>not</u> to enter goal compartments, rather than increase the number of entries made, as had been anticipated.

ii. Arena preferences

The times spent by the pups in the vicinity of the two goal box doors had been recorded, and the preference shown by each pup noted. These are shown in Table 14. Eight pups spent the total test period in the triangular part of the arena and are not included in the Table or analysis. Pups entering goal boxes were recorded as preferring that particular side, although in just one or two cases they actually spent less time on it than on the other.

Table 14

Comparison of numbers of pups from each group spending more time on one side of the open-field than the other

TREATMENT	PREFERRED SIDE		N	p
	Maternal	Nulliparous		
Group 1 (familiarised with apparatus)	19	16	35	0.610
Group 2 (Handled)	24	12	36	0.0318
Group 3 (Disturbed)	24	10	34	0.0264
Group 4 (Standard)	40	24	66	0.0614

The values in the column headed "p" are two-tailed probabilities obtained by using the binomial test.

With the exception of the pups which had been familiarised with the apparatus, there was some indication that they did prefer to be in the vicinity of the maternal odour, rather than spend their time near the non-maternal goal box. The effect is so slight, however, that it is probably best regarded as a "preference", rather than evidence for an "attractant" present in the excrement.

iii. Ambulation scores

Table 15

Mean Ambulation scores obtained from pups which spent their full testing time in the arena area

TREATMENT	MEAN NUMBER OF LINES CROSSED	N	p
Group 1 (familiarised with apparatus)	12.24	25	0.05
Group 2 (Handled)	20.18	28	0.001
Group 3 (Disturbed)	14.73	23	0.05
Group 4 (Standard)	5.66	42	ns

As the data met the criterion for homogeneity of variance, a t test, using the mean for each litter (after Abbey and Howard, 1973), was carried out between the scores obtained by the three groups given daily treatment and the standard pups. The probability values obtained, two-tailed test, are indicated in the final column of the table.

Animals which had not been disturbed between culling and the day of testing (Group 4), had the lowest ambulation scores. Intermediate scores were obtained from those animals which had been placed in the apparatus daily, or merely disturbed during the removal of the mother, whilst the handled pups were about four times more active than the standard group. It was observed that the high ambulation scores in Group 2 were typical of all members, not due to the hyperactivity of a small proportion.

iv. Conclusions

The overall conclusions drawn from these sets of data were that with the exception of the handled animals, which preferred the arena to the goal boxes, the other groups of pups behaved as if the apparatus did not contain any stimulus material in the goal compartments.

However, whilst the animals which had been familiarised with the apparatus failed to show any sign of preference for either the maternal or non-maternal excrement, the handled animals showed a slight preference for the maternal odour. Handling them appeared to affect their exploratory behaviour, as they were extremely active in the arena. The pups which had been disturbed (Group 3), and the standard pups (Group 4), also showed a preference for the maternal goal box door area, albeit only slight in the latter case.

Discussion

The difference in behaviour between animals which had been handled or disturbed, and those which had not - as evidenced by the number of goal box entries made and their activity in the arena, confirmed the earlier impression that the olfactory discrimination apparatus is a complex piece of test equipment to use. The pups' behaviour cannot be interpreted easily, as they are not merely making a simple discrimination, but are reacting to the complex situation of being removed from the home cage and placed in a somewhat stressful novel environment. In the absence of an attractive odour which compels the pups to move towards its source, their behaviour is highly variable.

The results obtained from the undisturbed group (Group 4), run under standard test conditions, can be compared with those obtained from all the groups tested in Experiment 4. In Experiment 4, 32.3% of the pups chose the maternal excrement and entered that goal box, whilst it was only 23.6% in the present experiment. Although this drop continues the trend first shown in Experiment 4, that is, for a decrease in the number of maternal goal box entries, when the two sets of results are compared as wholes, i.e., 31: 24: 41 and 17: 13: 42, there is no significant difference ($\chi^2 = 4.04$; df = 2), thus showing the behaviour to be stable over time.

The other dependent variable measured, which related to excrement preference, that of proximity to the goal box doors, did suggest that discriminatory behaviour was present, with a slight preference for the maternal odour.

It was interesting to see that the pups which had been disturbed daily, merely whilst their mother was removed and replaced, did show some signs of similarity to the pups which had been handled or familiarised with the apparatus; also that handling seems to bring about changes in the pups' behaviour.

The problem of excrement collection, and that it constitutes a variable in its own right, has already been mentioned in Part II of

Chapter VI. Unfortunately, in the same way that considerations of time and space had meant that none of the four groups run in this experiment could be matched with their own control, it was also not possible to compare the behaviour of each group with stimulus samples collected by means of both metabolic and holding cages. It was decided, for this experiment, that the relative separation of urine and faeces to be gained through using metabolic cages, was desirable, for it had been hoped that the pups run in this experiment would demonstrate an unequivocal attraction towards a substance contained in the faeces. This may have been a wrong decision, as the results indicated that the excrement might contain substances with aversive properties.

Because these experiments were being undertaken in order to discover whether a maternal pheromone existed, time constraints meant the possible existence of substances with aversive properties could not be investigated further.

Conclusion

When animals had been familiarised with the bright, open apparatus, they showed no signs of stress on being tested, as had happened in previous experiments, but explored all parts of it in an active manner. With pups to which the apparatus was a strange experience, but which were used to being handled, the majority remained in the arena, exploring it, but showing a preference for the maternal goal box area.

Higher proportions of the two groups of pups which had not been handled or familiarised with the apparatus actually entered the goal compartments (perhaps because the darker area was preferable to the bright arena), although the entries they made did not favour the maternal side. In both groups, the pups failing to enter a compartment spent more time in the vicinity of the maternal than the nulliparous goal box, perhaps indicative of a preference for the maternally-derived odour.

Whatever olfactory messages were being transmitted by maternal excrement, one thing now seemed clear, namely that it did not contain an attractant pheromone. The next, and final, experiments in which the olfactory discrimination apparatus was used, were designed with a new assumption in mind; that the lactating mothers used in the work so far were not emitting the maternal pheromone. In subsequent experiments, it was decided to examine factors which might be affecting the mothers adversely, rather than look at the offspring. Also, excrement would be collected from this point onwards by means of the small holding cages, to reduce the likelihood of samples containing substances with mildly aversive properties.

CHAPTER XI

DIET MANIPULATIONS

INTRODUCTION

After eleven experiments had been carried out, the main conclusion drawn from the results was that although the rat pups which had been used were capable of olfactory discrimination, the lactating females were not emitting a pheromone which acted as an attractant. The next direction which the research took was to discover why these animals did not emit the pheromone, like their American counterparts.

Two possibilities had been considered for quite some time. One was that the particular strain of rat used in Hull did not emit the pheromone, and the other was that the diet used was not suitable for its production.

That strain differences in rats exist, and may be of considerable importance, is now widely recognised (Levine and Wetzel, 1963; McIver and Jeffrey, 1967; Morrison, 1973), and it seemed quite possible that an attractant which is supposedly dependent upon bacterial growth may well be a strain specific phenomenon. However, the expense of obtaining different animals for testing in the olfactory discrimination apparatus meant that this possibility had to be left until all others had been investigated and exhausted.

Another important difference between the rats used in this study and the American ones was that they were given different diets. Although there seemed to be no reason why the Oxoid diet used in Hull should not be suitable for pheromone production and emission, the variable of diet was considered important enough to examine.

Diet is of central importance in the model of pheromone emission put forward by Leon, and this will be described fully in Part I of this chapter. Experiments 12 and 13, in which diet is manipulated as the independent variable, are reported in Part II.

PART I

THE ROLE OF DIET IN MATERNAL PHEROMONE PRODUCTION

Before Leon's monograph appeared in 1974, there was no hint that diet was to be of central importance in research on the maternal pheromone. Interest had centred on humoral agents, and prolactin in particular was implicated in the model of pheromone emission propounded by Leon and Moltz (1973).

Early in his monograph, Leon describes caecotrophe, and identifies this type of anal excrement as the substance which contains the maternal pheromone. Leon's account of caecotrophe production and the part it plays in pheromone emission is summarised in the following passage taken from his paper.

> In addition to the feces, which occur in dry, black and well-formed boli, lactating females emit large quantities of a semi-solid light-colored, unformed material. This substance,

called caecotrophe, is formed in the caecum of rats (29) and the increased caecotrophe defecation is quantitatively reflected in the rise of volatile components of the maternal anal excreta.

. . . .

The caecum is a large structure in the rat pouching out at the junction of the small and large intestine and becoming enlarged during lactation (14, 29). A digested mass enters the caecum from the small intestine, and the material that is pushed into the distal section of the caecum serves as a substance for bacterial growth and when defecated is called caecotrophe. The material in the proximal section of the caecum is passed through the large intestine and anus as the familiar dry, black, formed feces. The caecotrophe passes through the large intestine with relatively little water loss and is eaten by adult rats directly from the anus (39). This practice is called coprophagy, or more specifically, caecotrophy. The caecotrophe then mixes with the food in the stomach and small intestine (29), where the bacteria contained in the caecotrophe are critical for effective diet utilization and nutrition (5, 30, 43). Harder (29) suggested that the caecotrophe "...must have a special aroma which is more attractive to the animals ..." if adults were to discriminate the caecotrophe from feces for caecotrophy. Virgin female rats consume virtually all of the caecotrophe that they emit, but lactating females that attracted pups produced large amounts of caecotrophe that was not consumed immediately.

The excess caecotrophe was one obvious difference between those females that did emit the pheromone and those that did not, and it seemed probable that the caecotrophe was the specific portion of the anal excreta serving as the substrate for pheromone emission.

Leon, 1974; p. 443.

When tested in the olfactory discrimination apparatus, Leon's rat pups preferred caecotrophe to normal faeces obtained from a strange mother, and they also preferred caecotrophe obtained directly from the caecum of a recently killed Day 16 mother to an empty goal box, thus demonstrating that the pheromone is produced at caecal level, and is not a secretion added to the faeces at a lower level in the large intestine.

When virgin caecal contents were opposed to an empty goal box in the test apparatus, 16 day rat pups were attracted towards them, thus suggesting that <u>any</u> adult caecotrophe might have attractive properties for the young. However, when caecal material from virgins was opposed to an approximately equal amount of caecal material obtained from Day 16 mothers, the latter was preferred by the pups. Leon suggests that there might be <u>two</u> attractants present in anal excrement (Leon, 1974; Experiment 2K).

After postulating that microbial growth in the caecum is the site of synthesis of the pheromone, Leon confirmed the theory by demonstrating that both maternal and virgin caecal contents lost their attractiveness when such growth was inhibited by administering antibiotics to the female animals. Leon concludes that as lactating female rats have enlarged caeca, and hence more caecotrophe than virgin rats, it is a quantitative, rather than qualitative difference which brings about the pups' preference for the maternal excrement.

Next, Leon examined the medium for growth of bacteria in the caecum, and suggested that raw carbohydrate, necessary for this, must be present in the diet for the pheromone synthesis to occur. When his lactating rats were fed a diet with the only type of carbohydrate present being sucrose - absorbed above caecal level, the bacteria were deprived of their growth medium and the pheromone production was inhibited.

Returning to hormones, Leon explains how these are linked with events in the digestive system. It had already been established (Leon and Moltz, 1973), that high prolactin levels are associated with maternal pheromone production and emission. In Experiments 4 and 5 of his monograph (1974), Leon shows how prolactin, through elevating food and water intake, is responsible for providing the excessive quantities of caecotrophe for excretion. After using marker dye in the diet fed to the lactating female rats, Leon demonstrates that the weanling rats consume their mothers' caecotrophe, and postulates that this is a mechanism whereby the pups establish the flora necessary for adult digestion within their own gut. He ends his paper by suggesting that if young are prevented from producing their own caecotrophe, they might continue to approach the maternal odour past the age of about 27 days when its attraction normally diminishes. Also, the approach response might appear in adult rats if they too are deprived of their own caecotrophe.

In his next publication, "Dietary Control of Maternal Pheromone in the Lactating Rat" (1975), Leon reports a series of experiments in which both quantity and quality of diet were manipulated.

When lactating mothers' intake of food was restricted, pheromone emission, as assessed by pups' preferences, was inhibited. "...the critical difference between lactating as opposed to virgin females is the increased eating stimulated by the elevated prolactin levels that are evoked by developing young." (ibid., p. 315.)

In the next series of experiments, in which quality of diet is manipulated, Leon reports that if from the 14th day of gestation mothers are fed either diet "A" or diet "B", then pups will not only approach their own mothers excrement in preference to that obtained from virgin female rats fed the same diet, but they also prefer it to that from a mother fed a different diet. If excrement samples obtained from a mother and virgin female previously fed the same diet were placed in the olfactory discrimination apparatus, and pups tested whose own mothers had not been fed this particular diet, but the alternative one, they failed to approach the maternal excrement. The maternal pheromone now appears to be diet specific, and as Experiment 3 (ibid., p. 316), demonstrates, is a function of familiarity on the pups' part.

The results of Leon's numerous experiments are difficult to evaluate. Although χ^2 values appear against every test result, the reader is never informed as to which comparisons were made to obtain them. The only test which approaches that of a control group is reported in Table 2 (ibid., p. 314), where young are given the choice between excrement obtained from two nulliparous females. A non-significant χ^2 value is given for the division of 9: 13: 38 made by the 60 pups tested, but as Leon apparently compared it with another set of results, he does not appear to regard it as a group which can itself be used for comparison purposes. This problem has already been discussed in Chapter VI.

Because a great number of comparisons are reported, it is difficult to follow the thread of Leon's research. At times he sets up experiments which purport to test a particular hypothesis, yet often the data obtained are not suitable for the conclusions drawn. Inconsistencies between certain results are evident, and some of the assumptions inherent in the model of pheromone emission are suspect. These will be covered more fully in Chapter XIV, where Leon's work is considered both on its own merits, and in the light of the experimental work reported in this thesis. It is sufficient to say at this point that by the end of his paper on dietary manipulations, Leon has abandoned the view that the maternal pheromone is an innate species-typic releaser, and now regards the approach response as a learned response on the part of the pup to a specific odour emitted by the mother. The three models of associative learning, olfactory imprinting or familiarisation which he tentatively advances as competing explanations, were described in Chapter II.

Whatever view Leon takes of the ontogony of the pups' response to the maternal pheromone, the maternal diet is considered by him to be of central importance. It was to this variable that attention was turned in the next two experiments, in the hope that pheromone emission could be brought about in the PVG/C rats studied in Hull.

PART II

EXPERIMENT 12: THE EFFECT OF DIFFERENT DIETS ON PUP ATTRACTION TO MATERNAL ANAL EXCREMENT

Introduction

The American rats emitting the maternal pheromone were fed on Purina Rat Chow. An obvious step, after the failure to replicate Leon and Moltz's work, would have been to feed the rats in Hull on the same diet. Unfortunately this was not a practical proposition. Although a Purina diet is available in England, it is not the same as the American one, as it is manufactured from ingredients produced in Europe. The cost of obtaining the American diet would have been prohibitive, and in view of financial constraints its purchase could not be justified, particularly in view of the fact that it did not appear to differ in any important way from several standard laboratory rat diets available in this country.

The manufacturers of the Oxoid 41 B diet (Herbert Styles (Bewdley) Ltd.) had been approached to find out whether their diet met the carbohydrate requirements for pheromone emission, or contained an antibiotic. When several weeks had elapsed since the first approach was made, and a second letter also failed to elicit a response, the firm was contacted by telephone. A spokesman disclosed that the diet did contain sources of carbohydrate other than sucrose, and that there was no antibiotic added. He added that the firm would not divulge the constituents of the diet because they did not wish its composition to become known to other manufacturers of animal feeds.

Meanwhile, the Christopher Hill Group Ltd., manufacturers of the Labsure diets, proved much more helpful. They were in a position to be able to make an informed guess as to the contents of the Oxoid diet, and suggested that it resembled their PRD diet. Although there appeared to be no reason why either of these diets should inhibit pheromone production, a spokesman suggested that a new diet (Labsure CRM), manufactured for breeding animals, and which contained more starch than the normal diet, might encourage caecotrophe production. Whilst the PVG/C rats used in the experiments so far showed a three-fold increase in food intake (see Figure 3, p. 83), which was considered to be sufficient for the excess caecotrophe production needed, a diet which might increase this production further was worth trying. Leon's work on diet restriction (1975), indicated that pheromone emission was not the all-or-none affair postulated in the earlier paper (Leon and Moltz, 1972), but that it varied in direct relation to the quantity of food consumed. In this experiment then, the intention was to give the rats different diets, one of which had a higher starch content, known from Leon's work to be conducive to pheromone production and emission. The constituents of the two diets used, Labsure CRM and Labsure PRD, are given in Appendix I.

Purpose

Experiment 12 was carried out in order to determine whether PVG/C rats fed on the Labsure CRM or PRD diets would emit the maternal pheromone.

Subjects

56 PVG/C rat pups were used from 9 litters which had been culled on Day 0 to 6 pups, with the exception of one litter which comprised 8 pups. Females had been mated at 95 ± 10 days, and excrement samples were obtained from 6 nulliparous females of the same age. Pups were tested on Day 17. The 36 pups run in Experiment 3 provided data which were to be used for comparison purposes.

Apparatus

The olfactory discrimination apparatus, with an airflow rate of 29 litres/min was used for testing the rat pups.
Excrement was collected from adult animals by means of the small holding cages described in Part I of Chapter III.

Procedure

One week before the expected date of parturition, female rats were moved to one of two holding rooms, and changed from the Oxoid diet to either the Labsure CRM or PRD diet. All the mothers in a particular room were fed the same diet, along with three virgin females which were housed individually in the same room.

On Day 17, pups were tested in the olfactory discrimination apparatus, using the standard procedure, in a third room. They were given the choice of equal amounts of excrement obtained from either their own mother, or mixed from the virgin females housed in the same colony room.

Pups were deprived of maternal attention for 3 hours before the testing session began, whilst the adult females were isolated in the holding cages for excrement collection. The virgin females were isolated for 4 hours, and the mothers for 3 hours.

Results

The numbers of goal box entries made by the two groups of pups are given in Table 16.

Table 16

Goal box entries made by 17 day PVG/C pups whose mothers were fed the CRM or PRD diets

GROUP	PUPS' BEHAVIOUR			N
	Entered maternal goal box	Entered virgin goal box	Remained in arena	
Mothers fed CRM diet	5 (19.2%)	5 (19.2%)	16 (61.6%)	26
Mothers fed PRD diet	9 (30%)	7 (23.3%)	14 (46.7%)	30

χ^2 tests were carried out between the groups of results and the averaged control results of 9.5: 9.5: 17 obtained in Experiment 3. Both values of χ^2 were nonsignificant, being 1.24 and 0.136 for the CRM and PRD groups respectively.

The preferences shown by the rat pups for the different parts of the arena did not indicate any attraction towards the area nearest to the maternal excrement. The conclusion drawn from the data was that female rats fed either the Labsure CRM or PRD diets did not emit the maternal pheromone.

Discussion

The results of this experiment indicate that the two Labsure diets were no more conducive to pheromone emission than the Oxoid diet had been. Excrement from the female rats fed the PRD diet was distinguishable (by humans) from that derived from animals on both the Oxoid and CRM diets; it was moister and of a lighter colour. Although equal amounts of excrement were placed in the goal boxes, there was a possibility that the PRD diet, because of its higher moisture content, had a stronger odour than the virgin excrement, and for this reason attracted a greater number of rat pups. This small preference for the maternal excrement was no more than slight however, and is no more than might have been obtained by chance.

EXPERIMENT 13: RAT PUPS' DISCRIMINATION BETWEEN MATERNAL EXCREMENT AND THAT OBTAINED FROM FEMALES FED A NON-MATERNAL DIET

Introduction

Human observers were able to detect qualitative differences between the excrement obtained from rats which had been fed different diets. It seemed reasonable, in view of this, and also as Leon had demonstrated that familiarity with a particular odour was of more importance than a pheromone releaser effect, that rat pups, given the choice betweem their own mother's excrement and that obtained from a non-lactating female fed a different diet, should prefer the maternal odour. Although Leon had shown that pups preferred the anal excrement from a strange mother fed the same diet as their own mother, to that obtained from a strange mother fed on a different diet, it was decided to opt for an easier discrimination for the pups in this experiment, namely the choice between their mother's excrement and that obtained from a non-lactating female fed a different diet. As familiarity with general colony odour affects the pups' approach responses (Leon, 1975), it should be emphasised that animals fed different diets were housed in separate rooms, and testing was carried out in a room in which no animals were housed.

Three diets producing very different kinds of anal excrement were to be used. These were; the Oxoid diet, the Labsure CRM diet, and the Oxoid diet supplemented by sunflower seeds. Rats are extremely fond of sunflower seeds, and when eaten daily, they cause the animal to excrete very dry, black faecal boli.

Purpose

The aim of Experiment 13 was to determine whether 17 day old PVG/C rat pups would prefer to enter the goal box containing maternal excrement, when the alternative was one containing excrement obtained from virgin females which had been fed a different diet to the pups' mothers.

Subjects

78 PVG/C rat pups were tested on Day 17. They were obtained from 13 litters born to nulliparous females which had been mated at 100 ± 10 days, and which were culled to 6 on Day 0.
Excrement samples were obtained also from 9 nulliparous females of the same age as the mothers.

Apparatus

The olfactory discrimination apparatus, with an airflow rate of 29 litres/min was used for testing the rat pups.
Excrement was collected from adult animals by means of the small holding cages.

Procedure

As in Experiment 12, mothers were changed from the Oxoid diet to the new diet approximately one week before parturition.

Three groups of litters were formed, and each was given either the Oxoid diet, the Oxoid diet plus sunflower seeds, or the Labsure CRM diet. The three groups of rats were housed in separate rooms, together with three nulliparous females given the diet appropriate to the room. These diets were used until the pups had been tested in the olfactory discrimination apparatus, following the standard procedure, on Day 17. Pups were deprived of maternal attention for 4 hours before testing started; during this period, excrement was collected from the mothers and nulliparous rats by means of the holding cages.

In the test, pups were presented with equal amounts of anal excrement paired in the following manner:

Group 1	Oxoid pups	Maternal _versus_ Nulliparous fed CRM diet
Group 2	CRM pups	Maternal _versus_ Nulliparous fed Oxoid plus sunflower seeds.
Group 3	Oxoid plus sunflower seeds	Maternal _versus_ Nulliparous fed Oxoid diet

Results

Goal box entries are given in Table 17. χ^2 values are also shown; these were obtained by comparing each group's results with the averaged control results of 9.5: 9.5: 17 from Experiment 3.

Table 17

Goal box entries made by 17 day old pups, given choice of excrement obtained from females fed different diets

GROUP	PUPS' BEHAVIOUR			N	χ^2	p
	Entered maternal goal box	Entered virgin goal box	Remained in arena			
1 Oxoid	9 (37.5%)	CRM 5 (20.8%)	10 (41.7%)	24	0.980	ns
2 CRM	17 (56.7%)	s.seed 11 (36.7%)	2 (6.6%)	30	13.685	<0.005
3 Oxoid + s.seeds	12 (50%)	Oxoid 3 (12.5%)	9 (37.5%)	24	3.887	ns

It can be seen that only one group differed from the control, the pups whose mothers were fed the CRM diet, given the choice of her excrement opposed to that of nulliparous females which had been eating Oxoid pellets plus sunflower seeds.

Once more, there is a slight preference shown by all groups for the maternal rather than non-maternal excrement. When numbers of goal box entries only were compared with the 50:50 division which would be predicted by the Null Hypothesis, it was found that for Group 3, the choice of maternally derived stimulus material was significant (p = 0.036; two-tailed binomial test).

As in the previous experiment, there was no indication from the times spent by the pups in the various parts of the open-field that they preferred to be in the proximity of the maternal odour.

Discussion

Although at all times there is a slight preference shown by the pups for the maternally derived excrement, only one group demonstrates a preference which is statistically significant. In view of the evidence for a slight preference only, it seems safe to conclude that the pups are not drawn towards an attractant, but that they do perhaps approach a familiar, rather than unfamiliar odour. The possibility that an unfamiliar odour gives rise to avoidance reponses cannot be overlooked however. Experiments were not run in which pups were tested with excrement present in one goal box only, although Leon used this asymmetric presentation quite often (Leon and Moltz, 1971; Leon, 1974). It could be argued that the pups would show preferences for a goal box containing any slightly familiar odour to an empty compartment, regardless of whether or not there was any pheromone present. To test for aversive properties of stimulus material, it would have to be contrasted with an empty goal box, for results to be unequivocal.

As was suggested in Chapter IV, the amount of excrement present in the goal box (and probably its moisture content) might be likely to influence pups' selection. That the pups faced with an odour from moist excrement obtained from their own mothers, versus some very dry small non-lactating females' boli should choose the maternal sample, does not seem surprising. Indeed, it would have been more surprising if the pups had failed to make a discrimination when such gross differences in stimuli existed, although with Group 2, many pups did enter the goal box containing the drier excrement.

Although excrement had been collected by means of holding cages rather than metabolic cages, the trend evident since Experiment 4, for fewer pups to enter goal boxes, was not reversed. With the exception of Group 2, just less than half the pups tested remained in the arena, rather than enter a goal box. This weakens the hypothesis that animals held in metabolic cages excrete an aversive substance. It could be the case, however, that the animals tested in Experiment 2 were not typical, but for some unknown reason demonstrated a greater attraction towards the excrement.

During these experiments on diet, careful 24 hr observations of some animals were made, to determine whether they were eating all their caecotrophe as soon as they excreted it. There appeared to be very little difference between the lactating and non-lactating females, the former failing to consume a large proportion of their moist excrement.

Conclusions from Experiments 12 and 13

Feeding the PVG/C rats diets known to differ in composition from the Oxoid diet used in the previous experiments did not bring about the emission of the maternal pheromone in lactating rats. When rat pups were given the choice between excrement which was derived from their own mother and that from virgin females fed a different diet, they showed a slight preference only for the maternal odour - a result which had occurred in several previous experiments. This preference may be a function of familiarity, rather than due to the attractant powers of a pheromone.

CHAPTER XII

THE MATERNAL PHEROMONE IN WISTAR AND SPRAGUE-DAWLEY RATS

INTRODUCTION

Throughout the experimental work reported in this thesis, the possibility that the maternal pheromone might be strain specific loomed large. Leon and Moltz and their colleagues used Wistar rats, whilst the ones used in the Psychology Department's own colony at Hull University were a hooded variety.

In response to a letter written to Leon asking for information not given in his publications, and also mentioning the problems encountered with the rats tested at Hull, Leon sent a letter (dated 28.5.76) which can be seen in Appendix III. He mentions work recently undertaken at McMaster on a different strain of rat, and states " The hooded pups are also less deliberate in their choice than Wistars. They tend to run wildly in the test situation and thus increase the noise in the data. Their response can be altered by daily handling." In a later letter however, dated 24.8.76, Leon claims that hooded pups are "highly reactive" to anal excreta, and says that he fails to understand why his results cannot be replicated. He refers to a paper by Galef and Heiber in which hooded pups are tested; this was in press at the time he wrote, but was published in the same month, and is reviewed in Chapter XIII.

Whilst Experiment 11 was being conducted, a paper by Holinka and Carlson was published (Holinka and Carlson, 1976), in which they demonstrate the presence of the maternal pheromone in Sprague-Dawley rats, using a very slightly modified version of the olfactory discrimination apparatus. After finding some small differences between their rats and the Wistars run by Leon and Moltz, these writers conclude their General Discussion:

> Lactating Sprague-Dawley rats attract their pups by means of an olfactory signal, or maternal pheromone, emitted during a finite period after parturition. A similar type of olfactory-mediated pup attraction has been demonstrated in Wistar rats (Leon and Moltz, 1972). Its existence in two strains suggests that the maternal pheromone constitutes a general characteristic in laboratory rats.
>
> Holinka and Carlson, 1976, p. 502.

After Experiment 13 had been conducted, it was possible to take issue with this last statement, as there was no evidence that the maternal pheromone was emitted by the hooded PVG/C rats used in the Hull studies.

Having learned a great deal about the olfactory discrimination apparatus testing procedure through the experiments already carried out on hooded rats, it seemed to be a straightforward matter to test for the maternal pheromone in different strains; to this end, Experiments 14 and 15 were undertaken, and are reported in this chapter.

EXPERIMENT 14: THE MATERNAL PHEROMONE IN WISTAR RATS

Introduction

Fourteen pregnant Wistar rats were obtained from local breeders (Messrs. Bantin and Kingman), along with two nulliparous female Wistar rats of the same age. These animals settled into their new environment well, and the pregnant females gave birth to litters approximately one week after arrival without any problems.

Although Wistar rats are generally considered to be more placid than many other strains of laboratory rat, it seemed preferable to continue to use the small holding cage method excrement collection rather than metabolic cages, because of the possibility that the metabolic cages gave rise to the secretion of substances with aversive properties. However, because the holding cage method of collection did not enable good separation of faecal matter from urine, it was intended to sacrifice the mothers after 21 days and give the pups a second trial in the olfactory discrimination apparatus, using opposed maternal and virgin caecal contents as Leon had done in Experiment 2 K (Leon, 1974). Using caecal contents would also allow for the slight possibility that rats were consuming the portion of their anal excrement which contained the pheromone. Although this seemed unlikely, in view of the observations made on the animals, it could not be completely ruled out.

A new control group would have to be run with the Wistar rats, as it could not be assumed that they would behave in the test apparatus in the same manner as the PVG/C rats.

Purpose

Experiment 14 was carried out in order to determine whether the maternal pheromone was present in either the anal excrement or caecal contents of Wistar rats.

Subjects

72 Wistar rat pups were used from 12 litters born to nulliparous females aged 100 ± 10 days. Litters were culled to 6 on Day 0.

Excrement was collected from 2 nulliparous female Wistar rats, of the same age and origin as the 12 maternal rats.

Adult animals were fed the Labsure PRD diet from arrival in the Hull colony until the end of the experiment; maintenance was as described in Part I of Chapter III.

Apparatus

The olfactory discrimination apparatus, with an airflow rate of 29 litres/min was used for testing the rat pups.

Excrement was collected from the adult females by means of the small holding cages described in Chapter III.

Procedure

On Day 17, pups were tested in the olfactory discrimination apparatus after 3 hours maternal deprivation. The 12 litters were divided into an experimental and a control group. The experimental group pups were tested with excrement samples obtained from their own mother opposed to an equal quantity obtained from the 2 virgin females which had been isolated in the holding cages for 4 hours. Control group pups were run in the empty apparatus.

On Day 21, all the adult female animals were sacrificed, and their caecal contents used to provide samples for the goal boxes in the apparatus. Approximately 3 gm were used, and the pups were tested for the second time, with the caecal contents from their own mother opposed to those from the virgin female.

Testing took place over 2 days.

Besides noting the kind and number of goal box entries made, the time that each pup spent in the 3 divisions of the arena was recorded, and an ambulation score was obtained by counting the number of dividing lines crossed during test sessions.

Results

The goal box entries made by the Wistar rat pups on Day 17 and Day 21, are shown on Table 18. Probability values shown are the ones obtained from comparison of the experimental group results with the control, using χ^2 test.

Although there was still no evidence of an overwhelming attraction towards the maternal excrement or caecal contents, the pups were certainly influenced by the material in the goal boxes, in that the number of entries made was higher when it was present than when the apparatus was empty.

Table 18

Goal box entries made by 17 and 21 day old Wistar pups

GROUP	PUPS' BEHAVIOUR			N	p
	Entered maternal goal box	Entered virgin goal box	Remained in arena		
Day 17 anal excrement	15 (42%)	8 (22%)	13 (36%)	36	<0.001
Day 21 caecal contents	14 (39%)	6 (17%)	16 (44%)	36	<0.001
	CONTROL				
	Entered left hand box	Entered right hand box			
Day 17	0	1 (3%)	35 (97%)	36	

Of the goal box entries alone, the maternal material was chosen by the majority of pups in both groups. The binomial test carried out on these numbers gave non significant results (Day 17 pups, $p = 0.210$; Day 21 pups, $p = 0.116$; two-tailed values).

The mean ambulation scores of 11.5, 9.5 and 11.2 obtained from the Day 17, Day 21 and Day 17 control group pups which failed to enter a compartment, were indistinguishable. These pups did not prefer the part of the arena in the maternal goal box vicinity to any other part as shown by the time spent there.

Discussion

The control group results, in which 97% of the pups remained in the arena show how different these animals are to the PVG/C strain. They failed to show the signs of stress - such as freezing or darting, that the hooded animals had exhibited when placed in the arena, and their greater tranquillity was shown by their fairly high ambulation scores, which were similar to those obtained from the PVG/C pups which had been disturbed or familiarised with the apparatus in Experiment 11.

The number of goal box entries made by the pups which were tested with material present in the compartments was significantly higher than the number made by the control group. This does not necessarily mean though that the pups were attracted by a pheromone, and in fact the results barely differ from the 52%: 31%: 17% division obtained in Experiment 2.

In considering the actual goal box entries made by the pups, the preference for the maternally derived stimulus material does not differ from one which might have occurred by chance. Thus once again, the pups have shown a preference which is slight, rather than to the extent of being significant statistically.

When Leon (1974) gave 16 day old pups the choice between maternal caecal contents and virgin caecal contents (Experiment 2 K, Table 1), he found that 87% entered the maternal goal box and only 10% the one containing virgin caecal contents. The results of experiment 14 fail to replicate these particular findings he reports; they also do not suggest that the maternal pheromone is present in the caecum, and consumed by the animals as it is excreted.

The food intake of the Wistar rats was measured from Days 5 - 18 in the primiparous animals, and over a 10 day period for the virgin rats. The lactating females showed a three-fold increase in intake, the pattern being similar to that of the PVG/C rats (shown in Figure 3, p. 83), although it was on average, 13% higher. The virgin females consumed about 9% more food daily. The higher intake of these rats is probably because Wistars are larger than the PVG/C strain. It was considered to be sufficient for pheromone production (Leon, 1975; Leon, personal communication).

Conclusion

Although the Wistar rat pups did not appear to be as stressed by the test procedure as the PVG/C pups, they still failed to show anything other than a slight preference for maternal excrement or maternal caecal contents when these were opposed to the same type of stimulus material obtained from non-lactating female rats.

EXPERIMENT 15: THE MATERNAL PHEROMONE IN THE SPRAGUE-DAWLEY RAT

Purpose

The purpose of Experiment 15 was to determine whether there was any evidence of maternal pheromone emission in the Sprague-Dawley strain of rat, using young tested at 17 days age in the olfactory discrimination apparatus.

Subjects

45 Sprague-Dawley rat pups born to 7 nulliparous females which had been mated at 100 $\pm$ 10 days age were used. Litters comprised 6, 7 or 8 pups. Excrement was collected from 3 nulliparous females aged 120 days.

These animals were the normal stock animals used by the Department of Zoology, Hull University. Details of this strain are given in Part I of Chapter III; the animals had been fed the 41 B diet manufactured by Messrs. E.B. Bradshaw and Sons Ltd.

Apparatus

The olfactory discrimination apparatus, with an airflow rate of 29 litres/min was used for testing the rat pups.
Excrement was collected from the adult females by means of the small holding cages described in Chapter III.

Procedure

All pups were tested once at 17 days age in the olfactory discrimination apparatus, after 3 hours maternal deprivation. The 3 nulliparous females were isolated for 4 hours in the holding cages, so that their excrement could be collected, and the maternal females for 3 hours. Approximately 3 gm excrement obtained from a particular litter's mother, and that mixed from the 3 virgin females were placed in the goal boxes of the test apparatus.
The standard test procedure was followed; goal box entries, times spent in different parts of the arena, and ambulation scores were recorded.

Fifteen pups from the 7 litters were tested in the empty apparatus; these comprised a control group.

All the testing took place in one day.

Results

The numbers of goal box entries made by the Sprague-Dawley rat pups are given in Table 19.

Table 19

Choice behaviour of 17 day old Sprague-Dawley pups in the olfactory discrimination apparatus

GROUP	PUPS' BEHAVIOUR			N
	Entered maternal goal box	Entered virgin goal box	Remained in arena	
Experimental	5 (16.7%)	1 (3.3%)	24 (80%)	30
	Entered left hand box	Entered right hand box		
Control	2 (13.3%)	0	13 (86.7%)	15

The data do not satisfy the requirements for χ^2 test, as the cell frequencies are too small. If the numbers of goal box entries are pooled, although the control results still give rise to an "expected" frequency of less than 5 (Siegel, 1956), a test for the significance of difference between two proportions can be carried out (Bruning and Kintz, 1968). The result is $z = 0.793$; $p = 0.428$

(two-tailed probability), and so it is concluded that there was no evidence of any difference in behaviour between the pups tested with excrement samples and those run in the empty apparatus.

Although data from time spent in the different parts of the arena and ambulation scores were recorded, both dependent variables failed to suggest that there was any difference between the experimental and control groups, and so, as in previous experiments, for the sake of simplicity, details of these data are omitted.

Discussion

The results obtained from the Sprague-Dawley rats are very similar to those of the PVG/C rats run in earlier experiments. There is no evidence that they are attracted towards maternal excrement or spend their time in the proximity of the maternal odour, although once again, a slight preference for the maternal stimulus is shown.

The results did differ from the earlier ones, in that fewer pups from both groups entered either of the goal boxes. χ^2 test carried out on the control group from this experiment and from Experiment 3, using pooled cells and Yates' correction, gave a value significant at $p = <0.05$ level. ($\chi^2 = 5.266$; df = 1; comparison numbers 19: 17 and 2: 13)

Mean ambulation scores of 5.3 and 5.1 from the experimental and control group pups respectively, which failed to enter goal boxes, were similar to the score of 5.66 obtained from the PVG/C pups tested under standard conditions in Experiment 4 (Table 15).

Conclusion

Whilst the Sprague-Dawley pups tested in this experiment showed what was now coming to be regarded as the usual slight preference for maternal excrement samples in the olfactory discrimination apparatus, the results indicated that the behaviour of the pups tested in the apparatus with excrement samples present was indistinguishable from that recorded when the apparatus was empty. Thus Experiment 15 failed to produce evidence of the maternal pheromone being secreted in the Sprague-Dawley strain of albino rat.

Conclusions from Experiments 14 and 15

Despite the findings by Holinka and Carlson (1976), and correspondence from Leon (Appendix III) which suggested that the maternal pheromone was not a strain specific phenomenon, this possibility could not be overlooked. When two strains of albino Rattus norvegicus were tested in Experiments 14 and 15, there was no stronger indication of an attraction towards maternal anal excrement than had been evident in the hooded variety tested previously.

Whilst the Wistar and Sprague-Dawley rats used in Experiments 14 and 15 were undoubtedly "pure", having come from reputable dealers, and originally came from the same source as the North American animals, that common source probably existed about 50 years ago. Genetic drift would have occurred in the strains on both sides of the Atlantic, meaning that although the animals have the same label, in fact they might show quite different genetic characteristics, which may in turn be reflected in behavioural differences (Levine and Wetzel, 1963; Patterson, 1978). The conclusion must be, then, that although rats of the same nominal strain as the North American ones were tested in Britain, their failure to emit the pheromone could be attributed to "strain difference".

On the other hand, it might be that there are some specific bacteria present in the gut of the North American rats which cause maternal excrement to exert an attractant effect. To elucidate this problem, it would be necessary to compare closely the pheromone and non-pheromone producing animals, probably looking first at differences in gut flora. This would be a difficult, time-consuming and costly exercise, and was considered to be outside the scope of this study, the aim of which had been merely to replicate the maternal pheromone effect. Almost two years had been spent on this undertaking, which had proved unsuccessful.

The conclusion, after Experiments 14 and 15 had been run, was that whatever the reason or reasons, the failure to replicate the maternal pheromone effect indicates a real difference between the North American and British rats studied.

CHAPTER XIII

THE STATUS OF MATERNAL PHEROMONE BY MID-1977

INTRODUCTION

By early 1977, after several experiments with lactating female rats, there was still no evidence from the Hull laboratory that the maternal pheromone existed. The olfactory discrimination apparatus, used with success by Leon and Moltz, had been used throughout all the work, although with the incorporation of certain modifications in procedure, for the various reasons already given.

Whilst the experiments at Hull were being conducted, a certain amount of correspondence took place between Leon, Moltz and the present writer (copies of letters received are given in Appendix III). As mentioned in Chapter II, Leon and Moltz were, by 1977, working separately, and it became evident from some of the offprints which they supplied that they were pursuing diverging lines of research.

Both had expressed interest in the attempted replication and had offered their assistance. Consequently, the writer decided, in view of the total failure to obtain similar results, that a visit to Leon or Moltz and their laboratories would be a useful undertaking. Any differences in odour or composition of the maternal excrement could be discovered, and some of the problems encountered in the work on the maternal pheromone could be discussed fully. Soon after issuing an invitation to visit his laboratory, Leon became ill, and so a shortened visit to McMaster University was arranged before a longer stay with Moltz at Chicago University in April, 1977.

After 1976, several papers on the maternal pheromone appeared in the literature. Pheromones as agents in mammalian behaviour were being mentioned in textbooks (e.g. Hill, 1976; Hafez, 1975); the maternal pheromone in particular is described in the revised version of Barnett's authoritative work The Rat (1975), in the book by Stoddart (1976) Mammalian Odours and Pheromones, and in the general textbook Comparative Animal Behavior, (Dewsbury, 1978).

Chapter XIII summarises the understanding of the maternal pheromone held by mid-1977.

Part I comprises a brief recapitulation of the conclusions drawn from the experimental work in which the olfactory discrimination apparatus was used, in Experiments 1 - 15; in Part II, an account is given in diary form of the writer's visit to the two laboratories in North America. Finally, in Part III, pre-publication offprints which were made available to the writer at this time are outlined, together with other post-1975 publications on the maternal pheromone.

PART I

SUMMARY OF FINDINGS MADE IN EXPERIMENTS 1 - 15

The first experiment demonstrated very effectively that the standard procedure used by Leon and Moltz in obtaining odour preferences would have to be modified substantially in order to collect valid data. The use of live animals in the goal boxes gave rise to confounding variables, but the collection of uncontaminated faeces from lactating and non-lactating animals did not appear to be a simple matter either.

When 17 day old rat pups were given the choice of maternal versus non-lactating female excrement in the second experiment, just over half chose the maternal faeces - a figure far below that obtained by Leon and Moltz (1971), in a comparable experiment. Differences in apparatus and procedure which might give rise to this discrepancy were noted, and these were either amended or studied in subsequent experiments. The rate of airflow through the apparatus was one such variable, but the results of Experiment 4 showed that it did not in itself affect the rat pups' behaviour, although the preference of these rats for the maternal excrement did appear somewhat diminished when compared with previous results.

Lengthening the pre-test period of maternal deprivation did not elicit stronger approach behaviour from the pups, and pups tested at 24 days age also failed to move towards the source of maternal odour. However, these older pups did show a greater propensity to enter <u>either</u> of the two goal boxes as opposed to remaining in the arena.

The problem of pups which had remained in the arena (or "failed" to make a choice of stimulus), had been an important one, and the existence of a substantial number of pups in this group had given rise to difficulties over the interpretation of results. The solution appeared to lie in the setting up of control groups, in which pups were tested in the olfactory discrimination apparatus when it was empty. These provided a base-line against which overall behaviour in certain conditions could then be compared; the goal box entries alone could subsequently be examined for an indication of any preferences which may exist.

The results from Experiment 11 suggested that the pups' behaviour in the arena, and whether they actually made a choice, depended to a large extent on both their familiarity with the apparatus and the nature and extent of pre-test handling which had occurred. It appeared that the more familiar the pups were with the test procedure, the less likely they were to enter a goal box.

Metabolic cages had been introduced as a means of excrement collection, as the previous methods had not enabled good separation of faeces and urine. After Experiment 4, another variable was considered to be complicating the results. The results from this experiment suggested that excrement collected by means of these cages contained a substance,or substances, which had aversive properties for conspecifics. It was hypothesised that animals isolated in these cages underwent stress, and so secreted slightly different products from unstressed animals. However, although this may have been true for early sets of animals tested, later results were not in accord with this idea.

As it had been shown that the PVG/C rat pups tested were capable of olfactory discrimination, and that the apparatus was not malfunctioning, the conclusion that these particular animals were not emitting the pheromone seemed inescapable.

The same strain of rat was given two different diets in Experiment 12, in an effort to determine whether it was a dietary deficiency which was preventing the pheromone release, and in Experiment 13, three types of diet were used. Pups were given the choice of excrement samples which differed quite considerably in composition and odour, only one of which they were familiar with. The results were virtually unchanged; there was still no suggestion of any strong attraction towards the maternal excrement, although the slight preference for it, as opposed to the virgin excrement, which had been noticed in earlier experiments, was still evident.

The failure to obtain evidence of a maternal pheromone had at first been attributed to an apparatus fault or to some peculiarity of the pups tested. As more experimental work was completed, the fact seemed to be that the lactating female rats tested were not producing the maternal pheromone. There seemed to be no reason why the diet should block pheromone production, and feeding the rats on different diets had failed to alter the pattern of negative results. Finally, two different strains of rat were obtained and tested, Wistars and Sprague-Dawleys. These rats did not appear to emit the pheromone either, although the behaviour of the Wistar pups was markedly different from that of the PVG/C young.

Whilst it seemed legitimate to conclude that the maternal pheromone was not being emitted by any of the three strains of rat tested in the Hull laboratory, it was difficult to understand why this should be the case, if indeed maternal pheromone is "... a general characteristic in laboratory rats." (Holinka and Carlson, 1976, p. 502). The pheromone had been demonstrated to exist over a number of years by Leon and Moltz and their colleagues, working in two separate laboratories, and more recently in a third place by Holinka and Carlson. Whilst the constituents of the diet claimed to be necessary for pheromone production by Leon and Moltz were present in the British diet, without actually feeding the animals on the North American Purina diet, this variable could not conclusively be ruled out as the one which might be preventing pheromone emission.

A similar problem arose over the use of Wistar and Sprague-Dawley rats in the later experiments. Although the animals were derived from good stock, and originated from the same source as the American rats, the common source probably existed about 50 years ago. Genetic drift would undoubtedly have occurred in the strains of rat reared on both sides of the Atlantic, with the result that animals supposedly of the same "strain" might have different genetic and behavioural characteristics. To make detailed comparisons between the British and North American strains of rat, it would have been necessary to import animals.

Thus the conclusions drawn from the experimental work itself were of a rather limited nature. All that could be safely stated was that no evidence had been obtained for the existence of the maternal pheromone in three strains of British laboratory rat. However, there was an indication that the rat pups tested discriminated, and to a slight extent, preferred a maternally derived odour.

The experimental work had not been without value though. The attempted replications had shown up some inadequacies of experimental design which existed in the published reports. The failure to obtain evidence of an attractant meant that the original work was subject to close scrutiny - and this revealed inadequacies of a more theoretical nature. These will be described and discussed in Chapter XIV.

PART II

AN ACCOUNT OF THE VISITS TO THE PSYCHOLOGY DEPARTMENTS AT McMASTER UNIVERSITY AND CHICAGO UNIVERSITY

The Psychology Department, McMaster University, Hamilton, Ontario

Tuesday, April 5th 1977

This was the only day I met Dr. Michael Leon. The first question he put to me was as to whether or not I had obtained any positive results with my rats since my last letter to him. I told him that as far as I could see, my lactating rats were not producing caecotrophe, and that there was no behavioural evidence which suggested the existence of a "maternal pheromone". As he showed me round his animal colony, we considered the reasons which could be causing my apparent failure to replicate his work - the underlying assumption being that the phenomenon existed, but was being "masked" in some way by some other variable.

The main possibilities were:

1. I was feeding the mothers a diet which contained antibiotic.
2. My lactating mothers were not eating enough.
3. I was not using a suitable floor covering in the apparatus.
4. The airflow was not adequate.
5. The pups in the Hull colony suffered from a respiratory infection of some sort, and could not make olfactory discriminations.

As my pups are able to discriminate odour at 10 days age (Experiment 5), and entered goal boxes in the apparatus under some experimental conditions, I felt I could eliminate points 3 and 5. I had previously checked out the diet formulae for antibiotics, which were not present, and also recorded the mothers' daily food intake. The latter increased considerably over the course of lactation, and Leon agreed that the amounts they were consuming by Day 17 should have been sufficient for pheromone production. Unknown to both of us at that time, Moltz had demonstrated that hyperphagia is <u>not</u> a necessary condition however. Work on the apparatus (Experiment 4), had indicated that alteration of airflow rate did not have any effect on the pups' behaviour, and so points 1, 2 and 4 could also be discounted.

Leon mentioned the possibility that the Hull rats were producing caecotrophe at night and consuming it all then. As observational work had suggested that this was not the case, and as the pups tested with caecal contents in the goal boxes (Experiment 14) had failed to produce evidence of a pheromone, this explanation seemed unlikely.

A constructive suggestion made by Leon was that I should test pups in the dark. He has found that under these conditions they respond much more quickly to the maternal odour.

One of my early problems had been that of the animals "darting" rapidly into goal boxes. Leon suggested that I handle animals before testing, in order to deal with what appears to be an escape response. Leon and a colleague had encountered this tendency in hooded animals, but reduced it by handling and apparatus familiarisation sessions. I had already done this (Experiment 11), but found that this treatment reduced the pups' disposition to enter the goal boxes, so exacerbating the problem. Leon then told me that in some recent work, where they had again been handling pups before testing, they had also encountered this problem.

Caecotrophe

I was only able to see one female at the "correct" age for caecotrophe emission, i.e. 17 days post-parturition. She did produce a larger rounded mass of faecal matter in addition to the normal boli, but I was unable to distinguish its odour from that pervading the colony room.

Apparatus

Some differences between Leon's olfactory discrimination apparatus and the one built at Hull were noted. The major ones were:

1. The two walls between the open-field and the goal boxes were fixed in position, with a gap of 2.5 cm (one inch) above the inner ledge. This is not in accordance with the diagrams published by Leon and Moltz (Leon and Moltz, 1971; Moltz, 1975). In the Hull apparatus the dividing wall was made to swing inwards on a hinge, its lower edge 1.5 cm above the ledge.
2. The external walls of Leon's apparatus were transparent, whilst mine were opaque.

There are some consequences of these differences - for instance in the Hull apparatus large animals can enter the goal boxes when the door swings inwards. I felt very dubious about whether 60 day old rats could get through the gap in Leon's apparatus. The transparent sides on his apparatus mean that the animals will be more vulnerable to external cues, such as light or movement in the testing room. The olfactory discrimination apparatus in Leon's laboratory was placed in a separate testing room from the colony animals, but it was not positioned centrally under a single light source.

<u>April 6th - 8th, 1977</u>

I spent these three days in the Psychology Department at McMaster University with Leon's graduate students. I did not meet Leon again to talk to.

<u>Work in progress</u>

I was not given much information about current experimental work, but I understood the position to be as follows:

Leon is now viewing maternal pheromone as an "imprinting", rather than a learning phenomenon. (A learning hypothesis had been advanced in his 1975 paper "Dietary Control of Maternal Pheromone in the Lactating Rat") Consequently he is now exposing pups of different ages to different odours in order to determine whether there is any "critical period" effect; he has a paper in press which demonstrates that artificial odours can influence pup selection.

Leon is routinely using a diet manufactured by "Tertox" to give mothers who do not produce caecotrophe or maternal pheromone. Unfortunately I was not able to get the formula of this diet, but I believe that it is similar to diet S, which does not contain suitable carbohydrate for pheromone emission (Leon, 1974; 1975). I was unclear as to what the behavioural consequences on pups reared with these mothers were, or were meant to be, particularly as they were housed in the same colony room as the Purina fed mothers. Leon himself (1975), established that familiarity with an odour in housing conditions has important behavioural effects, and so must be regarded as an independent variable.

As far as Leon's work goes, the impression I received was that he is currently developing his "imprinting" hypothesis, and that maternal pheromone is primarily explicable in terms of innate processes. Moltz was briefly referred to; the direction his research is taking, in which the role of the liver is under investigation, was regarded as somewhat tangential.

I was not able to see normally reared rat pups tested in the olfactory discrimination apparatus. I was assured though, that the attractant effect of the maternal pheromone was so strong and reliable that an experiment involving testing young pups was now used as an undergraduate laboratory practical. However, as this is carried out using mobile animals in the goal boxes, thus confounding auditory and olfactory cues, I did not feel that the consistency of the effect, when obtained in this manner, should be regarded as evidence for the existence of the pheromone.

I left McMaster University feeling no wiser as to why my animals in Hull had failed to show the pheromone effect, and rather confused about the research Leon was currently pursuing.

The Department of Biopsychology, The University of Chicago

Monday April 11th - Saturday April 24th, 1977

During this thirteen day period I had many conversations with Professor Howard Moltz, and I worked with his two graduate students, Sally Kilpatrick and Kathi Marinari, every day.

The following general points emerged:

1. Caecotrophe

"Caecotrophe", as described by Leon, is not emitted by the rats in Moltz's laboratory (although Leon first noticed this phenomenon in Chicago), and the term is not used. Here, maternal rats do not produce two kinds of excrement, but either normal, large boli, or very loose, moist excrement of an almost diarrhoea-like nature. This picture is a familiar one, as the maternal females I have worked with produced similar types of anal excrement.

Although caecotrophe was not emitted, it did appear that between 14 and 27 days post-partum some lactating mothers produced faeces which had a distinctive odour. I was able to smell this odour easily, as it was very strong, and completely different from that of normal rat excrement. I was not familiar with this odour, and had not experienced it with any of the colony animals in Hull. Up to now, I had thought that caecotrophe was the moist type of excrement (only distinguishable by its looser constituency); after conversations with Leon and Moltz I no longer felt certain about what caecotrophe is, or looks like. Moltz dismisses Leon's hypothesis that the caecum can have two functions as ridiculous, and it certainly seems improbable that only a portion of the caecal contents should be subject to bacteriological change.

2. Failure of pheromone emission

Problems have arisen in Moltz's laboratory recently, in that not all lactating females are emitting maternal pheromone when tested by behavioural bioassay. So far, this failure of emission has been regarded more as an annoyance rather than a problem with serious implications, and no investigation has been undertaken into its spasmodicity. It is not known whether the loss of pheromone is associated with the unique odour, but certainly there seems to be no correlation between the pheromone and the quality of maternal excrement.

Sally Kilpatrick was attempting to filter out the mothers who apparently were not emitting the odour, by first testing the attraction they had for 16 day colony young. If three pups in succession chose the mother, then she considered that the mother was emitting the pheromone. However, Leon (1975) points out that exposure in a colony room to a mother emitting caecotrophe can influence a pup's choice in the apparatus, even if that particular pup has not been reared with a caecotrophe-emitting mother. The element of uncertainty which this finding introduces, together with the auditory and confounding variables present (mobile animals were placed in the goal boxes), means that these animals cannot be regarded as pheromone producers.

Moltz attributes this loss of pheromone emission to an inbreeding process. It turned out that he buys outbred Wistar rats from the suppliers every few months and then inbreeds from these animals. Recently a longer period than normal had elapsed without new stock, and bearing in mind a similar pattern of events in the past, Moltz suggested that the relevant gene had been bred out of the animals. He also recalled that a few years previously, some rats of the ACI strain had been purchased for another user in the Department, and when Leon, at the time Moltz's graduate student, had tested these rats for pheromone emission, he had failed to find it. This failure was viewed as an unimportant idiosyncracy of the strain.

3. Studies using wild rats

Moltz expressed concern over the failure of pheromone emission in my rats, and in view of his experience, seemed more ready to attribute it to some difference in the actual rats, rather than to some procedural difference or oversight, than Leon had been. We were agreed in that in investigating the pheromone, the ideal solution would be to work with wild rats; in fact I think that if selection pressures are to be considered as a serious factor, then the concept of a maternal pheromone in caged laboratory rats is rather odd. Such a means of uniting mother and young may be present in wild rats, and it may originally have been present in laboratory rats, as Moltz would like to think is the case with his newly purchased animals, but there would be no selective advantage in its remaining, unlike some other pheromones - for instance the "alarm" pheromone.

Even if a maternal pheromone is still to be found in some laboratory rats, then the precise mapping out of its first appearance and final wane can hardly be matched with events in wild rats in the way Leon postulates, for, as is amply documented by Calhoun (1962), life outside the laboratory proceeds at an entirely different pace.

4. Work in progress

At the time of my visit, Moltz was working on the hypothesis that attraction to maternal pheromone is really a "specific hunger" type of behaviour. The pups need to ingest maternal bacteria from the faeces in order to establish suitable microflora in their own gut - a process which takes several days. It is argued that if adult rats are caecectomised, so that they can no longer produce (and consume) their own caecotrophe, then they should display a preference for maternal excrement, high in caecotrophe content, and so the pheromonal effect is reinstated. Adult rats were tested in a new olfactory discrimination apparatus which had been built for this purpose. Like the first apparatus, it was not sound proof, and mobile animals were placed in the goal boxes. Some rats survived caecectomy, and after testing, which produced what I considered to be somewhat equivocal results, postmortems were carried out. These showed that the rats had not developed new caecal pouches.

I am critical of the method, results and interpretation of this experimental work for the following reasons:

1. Confounding of auditory cues in the apparatus.
2. Choice probabilities were not estimated either statistically or empirically by running animals with the goal boxes empty, or using test animals with peripherally induced anosmia. In effect, there is no control.
3. Although there was no sign that a caecal pouch had reformed in the animals which had undergone caecectomy, it could be postulated that bacteria in surrounding parts of the intestine might take over the caecal function. This would mean that the experimental animals were not really deficient at all.

Moltz had recently measured food intake on non-lactating concaveated females which emit maternal pheromone (as demonstrated by Leidahl and Moltz, in press), and found that hyperphagia is not a necessary condition for its production. Kathi Marinari is currently working with concaveated female rats, assessing maternal behaviour and blood serum prolactin levels at 21 days post-partum; it was not known what the prolactin levels of concaveated female rats would be.

Moltz's paper on the role of bile in pheromone production (Science, April 1, 1977) appeared whilst I was in Chicago. I found it extremely difficult to assimilate this development into the general framework, and I was grateful to Moltz for giving me a copy of his recent grant application form, in which he provides a research rationale and summarises his findings to date.

5. Apparatus

The olfactory discrimination apparatus was identical to that used by Leon at McMaster University, and the same criticism of the transparent sides can be made.

Testing was carried out in a room adjacent to the colony room, and a central fluorescent light provided good illumination.

When pups were tested, they were first examined for signs of respiratory infection, namely wheezy breathing. Any pups which had noisy breathing were not run.

Sally Kilpatrick remarked to me that sometimes, when virgin females were placed in the apparatus goal box, they would suddenly start to excrete faecal matter after the first pup had been tested, as if in response to pup stimulation.

- - -

During my stay in this Department, I had many useful discussions with Professor Moltz and his students, all of whom were extremely helpful, and willing to examine any aspects of the research which I cared to raise. However, I found some troublesome factors in the situation which I could not resolve to my satisfaction. These were:

1. The fact that live animals were used in the test procedure. Confounding variables were present, and on several occasions I saw a maternal rat "retrieve" a pup and pull it into the goal box.

2. The complete lack of control comparisons of any description.

3. The unreliability of pheromone emission. As maternal attraction (or the maternal pheromone) was being used as a tool in evaluating results of experimental procedures, I considered the fact that an unknown proportion of mothers were no longer emitting the attractive odour to have serious consequences for Moltz's data interpretation.

4. I found it difficult to believe that the 60 day young tested in the apparatus by Lois Leidahl (Moltz, Leidahl and Rowland, 1974), could enter the goal boxes with the door fixed in the way it was. Sally Kilpatrick shared my feelings on this matter. No one working in the Department at the time of my visit had actually seen these particular rats being tested.

5. I had the impression that although these researchers were only too well aware that not all their animals were emitting the maternal pheromone, this problem was of no interest to them.

I left the University of Chicago no wiser as to why the rats in Hull were not appearing to emit the maternal pheromone, and confused about the precise nature of caecotrophe.

However, after having seen testing carried out in the North American laboratories, I did feel more confidence in my own experimental procedure and results, and felt reasonably sure, on my return to Hull, that the rats I had tested had not been emitting the maternal pheromone.

PART III

THE LITERATURE ON THE MATERNAL PHEROMONE, 1976 - 1977

Whilst the experimental work reported in this thesis was being undertaken, a few more publications on the maternal pheromone in rats appeared; pre-publication offprints were made available to the writer by Leon and Moltz, and a report on some work carried out in Britain, which was never intended for publication, was discovered.

These papers are listed in Table 20, and a brief summary of each follows.

Table 20

Work on the Maternal Pheromone in Rats, 1976 - 1977

YEAR OF PUBLICATION	TITLE	AUTHORS & LABORATORY
1976	Pup Attraction to Lactating Sprague-Dawley Rats	Holinka & Carlson (New York)
1976	Role of Residual Olfactory Cues in the Determination of Feeding Site Selection and Exploration Patterns of Domestic Rats	Galef & Heiber (McMaster)
1977	Emission of the Maternal Pheromone in Nulliparous and Lactating Females	Leidahl & Moltz (Chicago)
1977	Bile, Prolactin, and the Maternal Pheromone	Moltz & Leidahl (Chicago)
1977	Establishment of Pheromonal Bonds and Diet Choice in Young Rats by Odor Pre-Exposure	Leon, Galef & Behse (McMaster)
1977	Dissolution of the Pheromonal Bond: Waning of Approach Response by Weanling Rats	Leon & Behse (McMaster)
Unpublished	Maternal Pheromone: Discrimination by Pre-Weanling Rats	Kendrick (Durham)

(i) Pup Attraction to Lactating Sprague-Dawley Rats

Holinka and Carlson, 1976

These writers investigated olfactory-guided behaviour in Sprague-Dawley rats using two kinds of test apparatus. One was a slightly modified version of the olfactory discrimination apparatus used by Leon and Moltz. The other, apparatus of their own design, still followed the general principle of an open-field with two goal boxes attached, but was specifically designed for testing the attractive properties of urine samples.

After having demonstrated that pups between 8 and 26 days age (inclusive), are attracted towards lactating mothers in the olfactory discrimination apparatus, they employ a litter substitution technique, similar to the one used by Moltz and Leon (1972), and confirm the finding that pheromone emission can be delayed and prolonged in the lactating females.

In order to demonstrate that pups' approach behaviour is mediated by olfactory, rather than any other cues, Holinka and Carlson repeated the basic procedure with 20 day old pups, but using goal boxes previously soiled by the lactating or non-lactating female adult rats. The maternal compartment was preferred by 89% of the pups. Using their own apparatus, Holinka and Carlson found that pups spent more time exploring the maternal urine compartment than the non-maternal. As they point out however, the urine was probably contaminated by faecal matter during collection in the metabolic cages, and should not necessarily be regarded as the source of the pheromone.

In general agreement with Leon and Moltz, the main differences arising from Holinka and Carlson's work are that mothers start to emit the maternal pheromone at 8 days post-partum, and that urine may have attractive properties. The earlier onset of pheromone emission (Day 8 post-partum, as opposed to Day 12) is attributed to a strain difference.

This paper concludes with the sweeping statement about the maternal pheromone:

> Its existence in two strains suggests that the maternal pheromone constitutes a general characteristic in laboratory rats.
>
> (Holinka and Carlson, 1976, p. 502)

(ii) Role of Residual Olfactory Cues in the Determination of Feeding Site Selection and Exploration Patterns of Domestic Rats

Galef and Heiber, 1976

Galef's main interest lies in feeding patterns in rats, rather than in the maternal pheromone. He had already published work on diet preferences in weanling rats (Galef and Clark, 1971b, 1972) and their movements towards feeding sites (Galef and Clark, 1971a, 1971b). In this paper, Galef and Heiber explore the possibility that young rat pups are attracted towards feeding sites because the mothers leave excrement there which contains the attractant, maternal pheromone.

After demonstrating in Experiment 1 that pups spend more time exploring an area of a feeding enclosure previously soiled by a lactating female rat than a clean part, Experiment 2 examines preferences for areas soiled by nulliparous females, lactating females fed the sucrose diet (said to inhibit pheromone production), and areas soiled with excrement obtained from lactating females in metabolic cages. The results indicated no difference in preferences.

As a different strain of rat (Long-Evans) has been used, Experiment 3 comprises a direct replication of Leon and Moltz's work on the maternal pheromone, using the olfactory discrimination apparatus, but with these hooded rats as subjects. Rat pups of 20 - 21 days age were tested; in the comparisons made, one of the goal compartments contained anal excrement of some sort and the other was empty. Samples of anal excrement were obtained from the pups' own mother, a lactating female fed the sucrose diet,and a nulliparous female. The results indicated that pups were attracted towards excrement obtained from <u>either</u> of the lactating females more strongly than towards the nulliparous females' excreta. This difference in attraction to females fed the sucrose diet, whilst having serious implications for Leon's explanation of pheromone production, is used by Galef and Heiber as an explanation for the failure to obtain the expected preferences in the first experiment. They attribute the difference in preference to a strain effect.

There is still the relative attractiveness of the area soiled by anal excreta of nulliparous females in Experiment 2 to explain, and Experiment 4 is an attempt to resolve this discrepancy. It is divided into two separate studies. The first repeats the testing in the olfactory discrimination apparatus with anal excrement, but with equal quantities of material taken from lactating and non-lactating females; the second looks at preferences for samples from nulliparous females when a tray, previously soiled by these animals, is placed in the goal compartments. The findings were that when excrement samples in the olfactory discrimination apparatus are equalised, the preference for the maternal excrement disappears, and the nulliparous females also excrete or secrete substances with attractive properties.

The remaining experiments reported return to the main area of investigation, namely the choice of feeding site, and are not of relevance to investigations into the maternal pheromone.

This paper reports findings which have serious implications for the explanation of maternal pheromone emission put forward by Leon, and casts some doubt on the strength of the pheromone effect which he and Moltz and their colleagues report.

First, the fact that the pheromone effect disappears when amounts of excrement are equalised in the olfactory discrimination apparatus suggests that its attractiveness may be a function of quantity rather than quality. Although Leon mentions (Leon, 1974; 1975) that lactating females excrete more than virgins, it is not known whether equal quantities of excrement were placed in the goal boxes of the test apparatus, as this is never mentioned specifically.

The attraction towards excrement obtained from lactating females fed the sucrose-based diet cannot be accomodated by Leon's model of pheromone production, and the finding that the young are also attracted towards substances obtained from nulliparous females is another serious difference.

From the data presented in Galef and Heiber's paper, it is difficult to extricate exact numbers of pups' choices, but it appears that in most of the comparisons made there are several pups which fail to enter a goal compartment. For example in Study 1 of Experiment 4, the writers report that 15 out of 48 pups tested (31%) fall into this category, hence the maternal pheromone certainly does not seem to have the same powers of attraction with these rats as is reported with Wistar rats.

The writers, with their interest in feeding behaviour, do not pursue the implications of their findings for the maternal pheromone. These are serious though, and strongly indicate that more careful investigations into its actual existence should be undertaken.

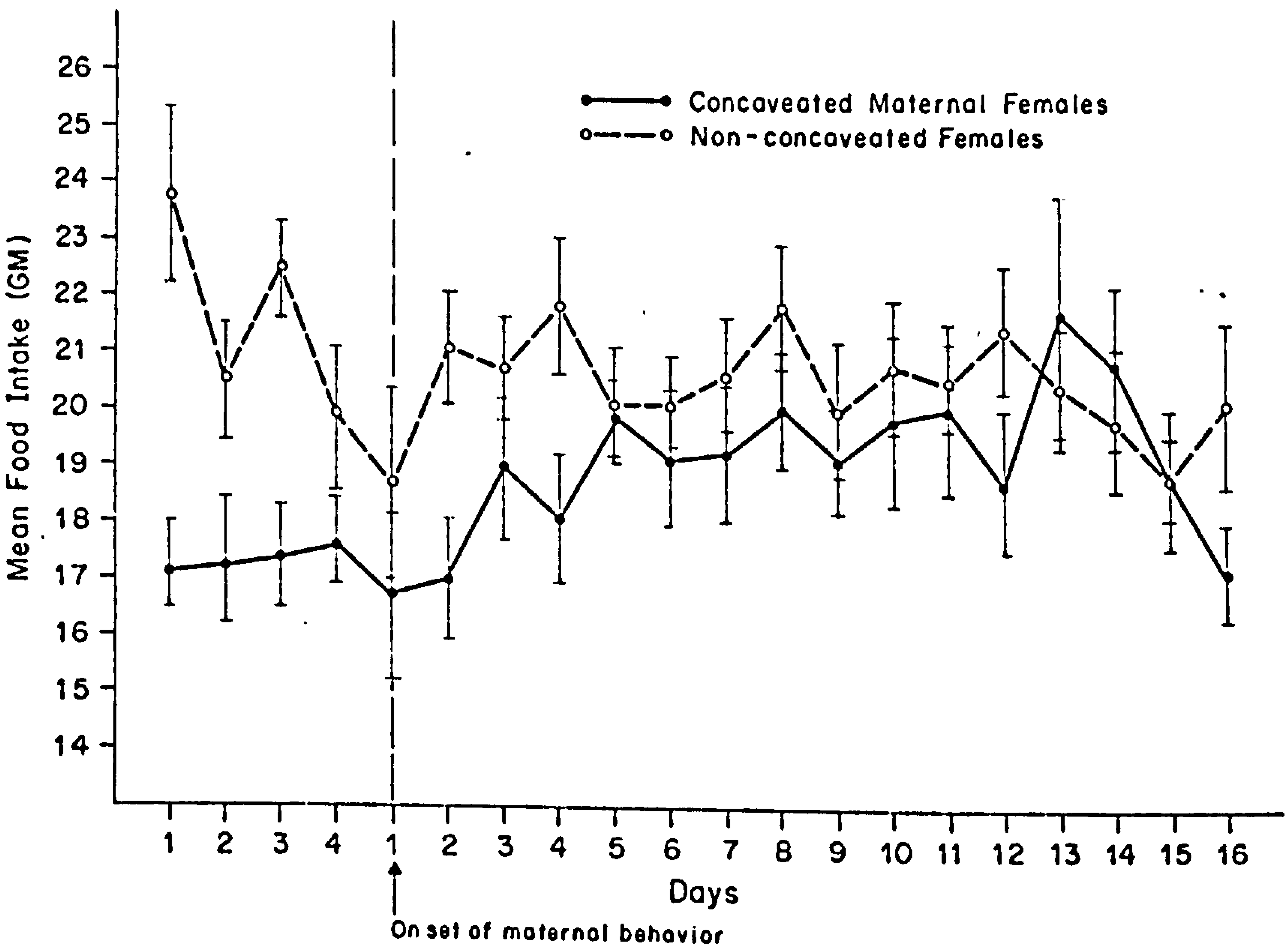
(iii) Emission of the Maternal Pheromone in Nulliparous and Lactating Females

Leidahl and Moltz, 1977

This paper is a continuation of investigations into concaveated animals, and the first experiment shows that normal 16 day young are attracted to faecal matter from such females, not merely to an odour which could be emanating from any part of their body. When faecal matter from concaveated maternal females was compared with that obtained from normal lactating animals, in Experiment 2, the standard pups failed to distinguish the two. In a third experiment, concaveated maternal females were injected with a prolactin inhibiting drug, and it was found that as with the normal parturient female, pheromone emission ceased.

Leon (1974) had proposed a model of pheromone emission in which high prolactin levels were associated with elevated food intake, and that it is the latter which increases caecotrophe production. In the fourth experiment reported in this paper, Leidahl and Moltz examine this model and find that concaveated maternal females eat no more food than do non-concaveated females, even though their faeces appear to contain the maternal pheromone. Thus the writers challenge Leon's model, in which hyperphagia is necessary for pheromone emission.

A slide of a graph, showing the mean daily food intake of concaveated maternal females, which is not included in this publication, but was given to the present writer when she was in Chicago, is shown in Figure 4.



Food intake of concaveated maternal and non-concaveated females; standard errors are indicated.

Figure 4: Graph of food intake of concaveated maternal and non-concaveated female Wistar rats (Courtesy of Professor H. Moltz)

(iv) Bile, Prolactin and the Maternal Pheromone

Moltz and Leidahl, 1977

Leidahl and Moltz (1975) had established that concaveated females but not successfully concaveated males emitted the maternal pheromone. Injection of prolactin or estradiol benzoate into concaveated males had failed to produce pheromone emission (unpublished data mentioned in this report), and led the writers to believe that a prolactin-hepatic mechanism was involved. In the female rat prolactin induces a much higher number of hepatic receptors than in the male, and so it was hypothesised that bile taken from a pheromone emitting female and injected into the caeca of male rats might give rise to pheromone production and emission. This exercise was carried out, and the results supported the hypothesis. It was also found that bile taken from a lactating female which did not emit the pheromone (either because she had only been lactating for 5 days, or because she had been receiving a prolactin inhibitor) did not render either the male animal, or his faecal matter attractive.

It is concluded that in the normal lactating female rat, a 16 day period is required for the formation of sufficient prolactin receptor sites on the liver to alter the content of the bile. Through this mechanism the biochemical environment in the caecum is altered, enabling the maternal pheromone to be produced.

(v) Establishment of Pheromonal Bonds and Diet Choice in Young Rats by Odor Pre-Exposure

Leon, Galef and Behse, 1977

In the publication written immediately before this one ("Dietary Control of Maternal Pheromone in the Lactating Rat", 1975), Leon presented evidence which suggested that the maternal pheromone is not an innate species-typic releaser. He postulated instead that the attraction of pups towards their mother's odour or a familiar maternal odour, may be due to olfactory imprinting, associative learning, or a non-specific familiarisation process. In the present paper, with some colleagues, he investigates these possibilities.

In Experiment 1, pups reared by non-pheromone producing mothers were exposed for 3 hours per day to air which had either been passed over a normal 16 - 21 day lactating mother, or filtered. Of the pups exposed to the maternal pheromone, 79% chose the maternal excrement when tested in the olfactory discrimination apparatus, whilst only 22% of those exposed to the filtered air made the same choice, suggesting that associative learning is not involved.

When the experiment was repeated, but exposure was to peppermint extract rather than to the odour of a maternal female rat, almost identical results reinforced the theory that mere exposure to an odour during early life is sufficient for an approach response to be shown by 20 day old pre-weanling rat pups.

Finally, pups from the second experiment were offered samples of peppermint or lemon flavoured diet for 24 hours on the day after they had been tested in the olfactory discrimination apparatus. Pups pre-exposed to the peppermint odour ate more of the peppermint-flavoured diet, unlike the controls, which preferred the lemon-flavoured diet. The writers conclude that the preferences shown by the test pups cannot be formed through association learning, but that the approach responses may indicate an aversion to novel stimuli. The influence of early olfactory experience on diet preference was demonstrated some time ago by Galef and Henderson (1972), Galef and Sherry (1973), and Capretta and Rawls (1974).

(vi) Dissolution of the Pheromonal Bond: Waning of Approach Response by Weanling Rats

Leon and Behse, 1977

In this paper, the decline of attractiveness shown to take place after Day 21 post-partum (Leon and Moltz, 1972) is examined more thoroughly. In the first experiment, pups were tested daily in the olfactory discrimination apparatus from Days 21 - 27 inclusive, and anal excrement, rather than live animals, was used as stimulus material in the goal boxes. The data obtained suggested to the writers that there is a "sharp" drop in responsiveness at Day 25. It is postulated that this decline could be due to the fact that the young are now producing caecotrophe themselves, and in the second experiment, Day 19 pups were tested in the olfactory discrimination apparatus with an empty goal box opposed to one containing the caecal contents of weanling pups of advancing age. The choices made by the pups indicated that it is not until 26 days age that weanling caecal material becomes as attractive as that obtained from adult animals. The results from the fourth experiment showed that if young are prevented from producing their own caecotrophe, then they are still attracted towards the anal excreta of lactating females at 30 days, an age when responsiveness has normally declined.

(vii) Maternal Phéromone: Discrimination by Pre-Weanling Rats

Kendrick, 1975

The basic aim of this experiment (submitted as a final year undergraduate dissertation), was to replicate Leon and Moltz's findings, using the olfactory discrimination apparatus, and testing 14 day old rat pups. In his version of the olfactory discrimination apparatus, Kendrick does not allow the pups access to the goal boxes, but only to an area immediately in front of their doors, through which vent holes allow the current of air to pass.

Two experiments are reported; the results from a preliminary one had been discarded as the apparatus was not free of a smell of paint, and also, as it had not been washed out after every trial. The first experiment was not successful. Of 14 pups tested, 5 went to the lactating mother and 9 to the virgin female, when live animals were used in the goal boxes. In the second experiment, trays containing anal excrement from maternal and virgin females were placed in the goal boxes, and the results were reversed. Ten pups now approached the maternal side and 4 the non-maternal side.

χ^2 tests indicated that neither of these results differ from a chance 50: 50 division; there were no non-choosers. The reversal of the preference obtained in the first experiment from the one expected, caused the writer some problems. His conclusion is that as the nulliparous females were more agitated by the test procedure than the maternal females, they may have given off a "fear pheromone", which somehow competed with the attractant given off by the mothers.

Why the pups should approach the nulliparous female in preference to the mother is not clear however. Kendrick suggests that perhaps it is the absence of an aversive factor, when the soiled trays only are used, which allows the preference for the maternal excrement to emerge.

Two points raised in this paper are of special interest. One is that the maternal females were not able to retrieve the pups in this apparatus, as there were no gaps between the open-field and the goal boxes through which the pups could move, or be pulled. Secondly, that a substance with aversive properties, a "fear" pheromone, should be postulated as one of the causes of non-replication.

Kendrick concludes:

> It would seem from the results of experiments 3a and 3b that the evidence found contradicts the findings of Leon and Moltz (1971). The same apparatus was used, but the results were completely different.

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These reports comprise the literature on the maternal pheromone in rats which appeared between 1975 and 1977.

Some work on the maternal pheromone in other small mammals was published; for instance by Porter and Doane (1976) and Porter and Etscorn (1976), on the spiny mouse. The most notable omission, that of studies on the mouse, was corrected by Breen and Leshner (1977) who demonstrated that the maternal pheromone is emitted in the house mouse, *Mus musculus*. However, as these researchers used mobile animals in a smaller version of the olfactory discrimination apparatus, their work was subject to confounding variables already described in connection with its use for rats. In addition, their results are somewhat equivocal.

Another piece of research was carried out in Britain. Another undergraduate's final year dissertation, it concerned the emission of the maternal pheromone in the mouse also. Using a modified version of the olfactory discrimination apparatus and an "improved" method, Knoff, a student at Keele University, undertook to replicate Leon and Moltz's work, using mice rather than rats. The apparatus was modified by placing the stimulus animals in a different set of boxes, joined individually to the normal goal compartments of the olfactory discrimination apparatus by rubber tubing. Air was passed over each animal, then into the goal boxes before entering the triangular open-field, and finally into the start box. It was assumed that keeping the stimuli animals away from the apparatus would control for "tactual, vocal and ultrasonic communication". Criteria for the choice of stimuli were either entry into the goal box, or spending most time outside one of the goal compartments.

Results from a number of comparisons using 17 day TO mice were fairly clear cut. The mice preferred compartments containing the odour of an intact lactating female mouse, or her anal excrement, and did not choose nulliparous females when these animals were paired against an empty compartment.

Information from Knoff's supervisor at Keele University, Dr. P Chevins, was sought regarding the rate of airflow through the apparatus.

It had been suspected at first that the current used in the Hull investigations was too weak. Knoff had used an airflow rate of only 9 litres/min however, and as this was perfectly adequate for the positive results he obtained (albeit with mice), it provided one of the reasons given in Chapter VI that the Hull apparatus was judged to be satisfactory in this respect.

Two more studies should be mentioned. One of them, by Hofer, Shair and Singh (1976), provided evidence that substances secreted by lactating female rats on their ventral surfaces stimulate suckling in the pups. The suckling response is mediated by olfactory cues. The other study was conducted on the heart rate of 10 day old rat pups by Compton, Koch and Arnold (1977). These researchers, using apparatus in which a current of air was passed over an anaesthetised stimulus animal, recorded in a relatively sophisticated way the effect that maternal odour has on the pups' heart rate. This technique is rather similar to the one used in a much earlier study by Schapiro and Salas (1970) (and also by Hofer, 1973), in which activity levels in young pups are recorded and found to be reduced in the presence of a maternal odour. Compton _et al_. found that 10 day rat pups which have been isolated for 2 hours, with their body temperature maintained, show a steady decrease of cardiac rate, which suddenly increases for a short period in response to the maternal odour. There is no increase shown by the pups presented with the odour of a non-lactating female, nor by the control group pups.

Studies of pyhsiological changes in response to maternal odour are not only interesting in their own right, but also of note because they use techniques which are more sensitive than the relatively crude behavioural bioassay. Unfortunately, these precise methods of measurement are not without their problems, perhaps the main one being the interpretation of vast amounts of finely detailed data obtained from the constantly running recording devices.

CONCLUSION

This chapter has provided a summary of events and publications up to mid-1977. The literature appearing since the start of the investigations in the Hull laboratory, whilst producing only one account of a replication of the maternal pheromone in rats from an independent source, seems in some cases to throw a few doubts on the generality and validity of the earlier findings.

A critique of both the pre-1975 literature on the maternal pheromone, and the papers reviewed in this chapter follows in Chapter XIV.

CHAPTER XIV

A SUMMARY AND CRITIQUE OF THE PUBLISHED WORK ON THE MATERNAL PHEROMONE

It has been found by Leon, Moltz and their colleagues, that Wistar rat pups are attracted towards the maternal pheromone, a substance emitted by lactating females, between 14 and 26 days age. Whilst pheromone emission can be prevented if mothers are exposed to newborn pups only, its appearance cannot be brought forward (Moltz and Leon, 1972). The attractant is found in caecotrophe, a soft type of anal excrement normally eaten by the adult rat, but produced in excessive quantities by lactating females (Leon, 1974), whose high prolactin levels (Leon and Moltz, 1973) are in some way associated with an increased food intake (Leon, 1974). That the young consume their mothers' anal excrement was also demonstrated by Leon (1974), and he postulates that this mechanism is the one whereby weanling rats build up gut flora appropriate for their adult diet.

Close examination of the effect of different diets leads Leon (1975), to suggest that the maternal pheromone is not an innate species-typic releaser, as he first supposed, but that pups are merely approaching an odour with which they are familiar. This hypothesis is borne out by the results of experiments in which rat pups are exposed to different odours (Leon, Galef and Behse, 1977), and in addition, such pre-exposed pups chose a diet which has a familiar odour. Finally, Leon and Behse (1977), demonstrated that pups cease to approach the maternal pheromone when they produce their own caecotrophe.

Moltz, Leidahl and Rowland (1974) had shown that mothers can continue to emit the pheromone until at least 60 days post-partum, although the period of attraction by the developing young could not be prolonged past 27 days. When female rats were successfully concaveated (i.e. displayed maternal behaviour after continuous exposure to young rats), they started to emit the pheromone. This is in contrast to females which failed to develop maternal behaviour after prolonged exposure to pups, and also to successfully concaveated male animals (Leidahl and Moltz, 1975).

The inability of males to emit the pheromone led to the hypothesis that emission was dependent upon chemical features of bile salts present in females only; subsequently Moltz and Leidahl (1977) demosntrated the importance of bile and prolactin for maternal pheromone production. Still working with concaveated animals, Leidahl and Moltz (1977) showed through blocking pheromone emission, that a single attractant exists in nulliparous concaveated and primiparous females; also, that increased food intake is not a prerequisite of pheromone production.

Independent confirmation of the existence of maternal pheromone came from one other source, Holinka and Carlson (1976), who showed that it acts as an attractant in pre-weanling Sprague-Dawley rats. As differences in pheromone emission were demonstrated to exist in Long-Evans rats by Galef and Heiber (1976), this finding casts some doubts upon the strength and generality of the effect obtained and reported in the literature.

The account of the maternal pheromone given by the North Americans sounds impressive, particularly as, over the years, a series of experiments has built up knowledge of the parameters and mechanisms involved in its production and emission.

Because the work carried out in Hull failed to replicate the published findings, these were not suddenly open to doubt, although they might cause one to take issue with the claims as to the generality of maternal pheromone emission, either across strains, or even within strains if genetic drift has occurred. However, replication attempts did show that the original work contained several serious design flaws, and subsequent examination of the publications revealed further inadequacies. A visit to two of the three laboratories where the maternal pheromone had been investigated only served to reinforce the impression that the phenomenon was not as robust as might appear from the literature.

In considering the literature on the maternal pheromone, the following points will be discussed in the remainder of this chapter:

I. Problems arising from, or associated with the use of the olfactory discrimination apparatus.

   A. Confounding variables present in the test procedure

   B. Use of excrement samples for test comparisons

   C. The need for a control group

   D. The unsuitability of this apparatus for testing olfactory discrimination in young pups.

II. Aspects of statistical analysis.

III. Problems of interpretation, and assertions which may be queried.

IV. Post-1975 differences between the findings of Leon and Moltz.

I. Problems arising from, or associated with the use of the olfactory discrimination apparatus

A. Confounding variables present in the test procedure

The most immediate consequence of replicating Leon and Moltz's work was the realisation that when live, mobile animals were used as stimuli in the olfactory discrimination apparatus, their presence provides four confounding variables; 1. auditory cues, 2. amounts of excrement defaecated in the goal boxes, 3. retrieval by maternal animals, 4. olfactory cues other than that of the maternal pheromone.

1. Whilst animals are kept in the goal boxes, they move about and may emit calls which are either audible to humans or of an ultrasonic nature. It is known that young pups will approach a source of noise, and possibly any of these types of auditory cue might bring about approach responses from the pups in the apparatus.

There was a marked difference in general activity level between the maternal and non-maternal females noted in Experiment 1, when the PVG/C mothers became very agitated. Kendrick (1975) found the opposite to be the case with his rats; the virgin females were more active in the test situation. Whether or not the activity is due to stress, and is associated with the release of substances with aversive properties is an open question. The only safe course is to avoid this possibility by not using animals as stimulus material unless they are immobilised in some way.

2. As lactating animals in the goal boxes produce more anal excrement than their nulliparous counterparts, the difference in quantity of anal excrement might determine preferential approach by the pups. The quality of the faecal matter might also vary in a systematic manner - and not only in that the maternal excrement contains a pheromone. It is also known to differ in consistency (wet weight), and because of this, it could give off a stronger odour.

3. The third confounding variable is that the maternal female rats, when approached by a young pup will retrieve it, whilst the nulliparous females will not. The result of this behaviour was clearly seen in Experiment 1 also, when all the pups tested in the olfactory discrimination apparatus "entered" the maternal goal box because they were pulled in by their mothers. So, for whatever reason the pups might have approached the mother initially, and it may or may not have been due to the attractant properties of the maternal pheromone, the design of the apparatus means that retrieval by the mothers can occur, ensuring that the young end up in her goal compartment.

4. Finally, it could well be the case that lactating females emit odours from their bodies, as well as from their excrement, which serve to attract the young. The work of Schapiro and Salas (1970),

Compton et al., (1977) and Hofer et al., (1976) certainly indicates that "body" odour is a variable which has direct behavioural consequences.

Once it was realised that the use of live animals in goal boxes through the concomitant confounding variables, gave distorted results, they were not used for any more comparisons in the Hull work. This inadequacy was apparently not considered to be of importance by the Americans, as a large part of their experimental work involves the use of mobile animals in comparisons. Just how large a part can be seen from Table 21, which lists the publications on the maternal pheromone together with information on the type of goal box contents used.

In the 12 papers written by Leon and Moltz and their colleagues, 208 test comparisons are reported. Of these, 134 involved using live, mobile animals in the olfactory discrimination apparatus. Holinka and Carlson (1976) provided independent evidence for the existence of the maternal pheromone, but in only 2 of the 25 comparisons they report are excrement or urine samples, as opposed to mobile animals used. Galef and Heiber (1976) used excrement samples only.

Thus two-thirds of the body of evidence on the maternal pheromone may be regarded as unsound, because of the confounding variables present when live animals were used for testing purposes.

Table 21

Type of comparisons made in published
studies on the maternal pheromone

AUTHOR	YEAR	NUMBER OF EXPERIMENTS	NUMBER OF COMPARISONS	NUMBER OF COMPARISONS USING:	
				EXCREMENT SAMPLES	MOBILE ANIMALS
Leon and Moltz	1971	1	8	1	7
Leon and Moltz	1972	4	32	0	32
Moltz and Leon	1973	2	4	0	4
Leon and Moltz	1973	2	7	0	7
Moltz, Leidahl & Rowland	1974	2	36	0	36
Leon	1974	7	41	27	14
Leon	1975	4	16	16	0
Leidahl and Moltz	1975	1	15	0	15
Holinka and Carlson	1976	6	25	2	23
Galef and Heiber	1976	2	7	7	0
Leidahl and Moltz	1977	4	13	2	11
Moltz and Leidahl	1977	1	14	7	7
Leon and Behse	1977	3	18	18	0
Leon, Galef & Behse	1977	2	4	4	0
TOTALS		41	240	84	156

It cannot be the case that the Americans were unaware of maternal rats retrieving young, because whilst the writer was in Chicago she witnessed instances of retrieval behaviour occurring during the test sessions with the olfactory discrimination apparatus.

The investigator running the rats (Sally Kilpatrick) confirmed that this was a fairly common occurrence, but pointed out that the pups could only be pulled into the goal boxes by the mothers when they were in close proximity, i.e. had virtually made their choice. It has already been suggested however, that rat pups might approach a lactating female for a variety of reasons, not necessarily because of an attractant contained in their caecotrophe.

This design fault is not restricted to work carried out on rats. In their investigation into the maternal pheromone in the mouse, Breen and Leshner (1977) also use live animals for one set of comparisons. They claim that there is evidence for the maternal pheromone in this species, even though they indicate an awareness of auditory cues providing confounding variables.

The olfactory discrimination apparatus can be modified, as it was by Kendrick (1975), so that there is no means of access from the arena into the goal boxes. Although this prevents the retrieval by the maternal rats from occurring, it does not eliminate the auditory confounding variables. This was done in Knoff's version of the apparatus (1975), in which the stimulus material was separated by some distance from the goal boxes and arena. Provided that the containers are sound proof, live animals could be used to provide olfactory stimuli without the pups being influenced by auditory cues.

B. Use of excrement samples for test comparisons

Whilst live animals are in the goal boxes, they produce excrement. Different quantities of this matter comprise a confounding variable, and it is known that the lactating female rats produce much more anal excreta than the virgin females. It is better to use excrement samples than mobile animals in the olfactory discrimination apparatus, but when this is done, the quantity must be equalised.

In approximately one-third of the comparisons listed in Table 21, excrement samples alone were used for testing. These comparisons are included in the 9 papers by Leon and Moltz (1971), Leon (1974, 1975), Holinka and Carlson (1976), Galef and Heiber (1976), Leidahl and Moltz (1977), Moltz and Leidahl (1977), Leon and Behse (1977) and Leon, Galef and Behse (1977).

In early replications at Hull University, when it had been intended to use excrement samples only, it was found that non-lactating rats often failed to produce any excrement at all during the 3 hour test period. Whilst this aspect of collection was never mentioned as a problem in any of the early American work, it is known from the data presented by Leon (1974; Experiment 1, Fig. 1) that the virgin rats he used excreted about 1 gm of faecal matter over a 3 hour period, in contrast to about 11 gm over the same time period, from 16 day lactating females. The information given by Leon in many of his papers is: "The anal excreta were collected from females isolated for 3 hr in a stainless steel box with an absorbent paper floor; the material was placed in a plastic weighing dish which was then put in either of the two goal compartments." (Leon, 1975, p. 315) It should be noted that there is no mention of equal quantities of excrement subsequently being used as stimulus material.

In the very first publication on the maternal pheromone by Leon and Moltz (1971), one of the several comparisons made was for preferences for goal boxes which had previously been occupied by a lactating or a nulliparous female. No details are given of the amounts of excrement present when the animals were removed. It could well be the case that the nulliparous female failed to leave behind any anal excrement, and that the maternal sample was approached simply because it had a familiar odour.

In the monograph "Maternal Pheromone" (1974, p. 442), Leon gives the standard account of excrement collection, but adds that after the collection period, excreta were removed with clean glass slides and weighed before being placed in the goal boxes. It seems that the excrement is weighed only because in this first experiment (where the account appears) the wet weight and volatile components of the excrement were determined. Again, there is no mention of equalisation of samples for test purposes.

Although in the next experiment, Leon states that the comparison results K on Table 1 are from "approximately equal quantities of caecal material from 5 day 16 mothers and 5 virgin females", this is the only instance in the whole paper (which contains 27 comparisons using excrement) where equalisation of amounts is mentioned. In his next paper on dietary manipulations (1975), in the 16 comparisons described, there is no indication that there is any control for quantity of excrement used; this is also the case in the paper by Moltz and Leidahl (1977).

The problem of equalising amounts of excrement when this is to be used as stimulus material in the olfactory discrimination apparatus is only raised explicitly by Galef and Heiber (1976). In their first experiment using this apparatus, they failed to note any differences in attractiveness of excrement obtained over a 3 hour period from lactating and sucrose-based diet females. They discovered that lactating females produce almost eight times more excrement than virgin females over a 3 hour period, and consequently they repeated the experiment, but using equal amounts of excrement in the goal boxes. In six out of the seven comparisons, Galef and Heiber placed anal excrement in one of the goal boxes only, and a clean container in the other. Their results indicated fairly strong approach tendencies by the pups, but this behaviour cannot safely be attributed to the maternal pheromone, as it may have been a function of familiarity with the odour of anal excrement. In the one comparison where equal amounts of anal excreta from two animals (nulliparous and lactating females) are placed in the two goal boxes simultaneously, 50% of each litter chose the maternal excrement. Thus there was no evidence of any pheromone being present.

Whilst this finding is in keeping with Galef and Heiber's investigation into feeding preferences, it is not easy to reconcile with Leon and Moltz's work. It is not known whether Leon and Moltz equalised the amounts of excrement used in many of their studies.

If they did not, it would seem that the maternal pheromone is largely an artefact arising from this particular confounding variable.

Leidahl and Moltz (1977, Experiment 1), state that in using faeces for testing, "Care was exercised to collect the same number of fecal boli from each animal." As both sets of animals used in this particular experiment were not lactating, there is no problem here, and this experiment may be regarded as a valid comparison. However, there is no mention of amount collected in Experiment 2 of the same paper, in which lactating females were compared to concaveated females, and it may be that in this comparison, unequal amounts were used. There is no reason to suppose that concaveated females excrete more than non-concaveated animals, as their food intake is no higher.

In the next papers to consider, By Leon and Behse (1977) and Leon, Galef and Behse (1977), excrement is often placed in only one of the goal compartments, and so the question of equalisation of amounts is not applicable. It should be noted that in these two papers however, a "strong" version of an innate attractant has been abandoned, and the investigators are looking at "preferences" rather than "pheromones".

Holinka and Carlson (1976), in Experiment 5, tested pups by giving them the choice of goal boxes previously soiled by their mother or a non-lactating female. There is no reason to suppose that the non-lactating control animals produced as much anal excrement as the mothers, and so this experiment also is open to the criticism that there was a failure to control the amount of anal excrement used as stimulus material.

In passing, it can be pointed out that in experiments where soiled goal boxes are used, there is of course the possibility that substances secreted in urine influence the pups' behaviour.

Leon (1974, Fig, 1) gives data showing that over the 10 - 21 day post-partum period the volatile components of maternal excrement increase, as does also its wet weight. This change was considered to reflect the concurrent change in caecotrophe and maternal pheromone emission.

It is possible that in many of the comparisons where excrement is used that the young would be attracted towards different types of faecal matter simply as a function of their consistency. Even if equal amounts of excrement were to be used in the olfactory discrimination apparatus for comparisons, preferences may not indicate any difference in composition, or presence of a pheromone, but merely moisture content. This problem shows the need for extremely careful investigation.

In summary then; of the 84 comparisons reported in which excrement samples are used as stimulus material, 36 are carried out with excrement in one goal box only; 45 use two samples, but it is not known whether equal quantities are compared; in only 3 is it explicitly stated that equal amounts of excrement are used. One of these is the comparison made by Leon in which caecal contents from Day 16 mothers and virgin females were compared (Leon, 1974; Table 1K). Leidahl and Moltz (1977, Experiment 1) showed that faeces of concaveated females were preferred to those obtained from non-concaveated animals, and the third comparison by Galef and Heiber

(1976; Study 1, Experiment 4) has already been described in detail; these researchers obtained no indication of preference for the maternal excrement.

Thus what appeared at the outset as an impressive body of evidence has now dwindled to evidence based on three test comparisons, one of which fails to substantiate the maternal pheromone effect.

C. The need for a control group

Early experimental work with the olfactory discrimination apparatus using rat pups which often failed to enter a goal box, necessitated the establishment of a control group to determine what proportion of pups might be expected to remain in the arena. It was argued in the third section of the Discussion in Chapter IV that whilst many of the early American experiments presented clear-cut results, as time went on the picture became more complex, partly due to the rising incidence of pups which did not enter a goal box. The need for controls for the analysis and interpretation of the data is evident, yet at no time do the North American investigators ever report results from animals run in the empty apparatus.

D. The unsuitability of the olfactory discrimination apparatus for testing preferences in young pups

The distance from the start box door to the goal boxes in the olfactory discrimination apparatus is 45 cm. Although it appears to be stating the obvious, it is essential that pups are able to travel this distance in order to gain a valid preference indication.

One day old pups are certainly unable to travel this distance, and the olfactory discrimination apparatus is unsuitable for testing such young pups, even when the stringency of criteria constituting a "choice" is lessened by reducing the distance requirement.

Very young pups also become cold and immobile if left untended by the mother, and unless special arrangements are made to keep their body temperature up. During the period of maternal deprivation, those pups younger than about 8 days age will probably suffer a certain amount of immobility in the absence of such arrangements, and so not give valid data in the test situation.

Holinka and Carlson (1976) found that the rats they tested showed an attraction to the maternal pheromone at the age of 8 days, 6 days before the Wistars tested by Leon and Moltz. They attribute this to a strain difference - yet it might simply have been that their rats were warmer when tested.

In a much more recent publication, Cornwell-Jones and Sobrian (1977) have used a preference testing procedure in which pups were kept warm on heated pads before being tested. The writers showed that Sprague-Dawley and Wistar rats as young as 3 - 4 days age are able to discriminate and prefer nest to clean pine shavings. They take issue with the claim made by Leon, Moltz, et al., that rat pups cannot discriminate at an early age, suggesting that it is the test procedure which is at fault. Of necessity they challenge the claim that young are attracted to a pheromone which is not produced by the lactating mothers until about 14 days post-partum.

Gregory and Pfaff (1971) summarise the need for care in selecting the appropriate behavioural tests when they state "These observations point out the importance of considering the response system used as a measure of performance in olfactory testing of young rats." (p. 575). In testing approach behaviour, the olfactory discrimination apparatus makes unrealistic demands upon young pups.

II. Problems of statistical analysis

A few omissions and problems are evident in the statistical analyses of results presented by the Americans. In the following discussion, the fact that most of the experiments contain confounding variables is ignored, and the results are taken at face value.

In the first paper on the maternal pheromone (Leon and Moltz, 1971), the reader is not informed which statistical test was used to obtain the probability levels quoted in the final column of Table 1 (p. 266). There is no information about the behaviour of pups which failed to choose the mother or maternal excrement (i.e. the number of pups remaining in the arena). When the data are reworked, using χ^2 test on an expected 50:50 division, the probability levels quoted, or ones exhibiting even greater significance levels are obtained. The 50:50 division is made on the assumption that none of the pups tested remained in the arena, forming a third category. However, in these comparisons the number of pups approaching the mother or maternal excrement is always high, and the existence of a few non-choosers would not alter the conclusions reached.

A "no choice" category appears in the paper "Stimulus Control of the Maternal Pheromone in the Lactating Rat" (Moltz and Leon, 1973), but again, the reader is not informed of the statistical test used to give the significance levels quoted. It has already been argued in Chapter IV that the nature of the experimental prediction should enable a precise statement of the Null Hypothesis to be made, against which the results can be compared. In the absence of both a statement and a control group, the reader is left to rework all the data in order to find out how the decisions as to significance or nonsignificance were obtained.

In this particular paper, only one significant result is reported; that for the lactating females which had reared foster pups of advancing age and were tested with standard 16 day young. As 106 out of 120 pups chose the maternal female, there is little doubt about the significance of these results.

In Table 1 (p. 70) of the same paper, three more sets of results are given, accompanied by the statement "Reference to Table 1 and Fig. 1 shows clearly the influence of such constant aged young in preventing the release of the maternal pheromone ($p>0.01$).", and later, "Thus, Experimental Group 2, given 10-day-old young shortly after parturition, did not evidence the pheromone when tested 6 days later ($p>0.01$)." Whilst it is "clearly" the case that there was no overwhelming preference for the maternal animal in Experiment 2, as 72 pups chose the maternal animal, 36 the nulliparous female and 12 made no choice, one might argue that there was <u>some</u> preference evident. When the animals making a choice (108) are compared with the expected 50:50 division, there is a significant difference ($\chi^2 = 6.16$, $p = < 0.02$). Certainly the writers state that the significance level was more than 0.01, but an obtained value of 0.02 is quite respectable. Leon and Moltz do not state why they set the α level at 0.01 for these results.

The statistical analysis just carried out on the results, using the 50:50 division, might be regarded as somewhat dubious, on the grounds that pups failing to enter goal boxes are not taken into consideration. However, a sound comparison can be made between the complete set of the first and final results given in the Table. These are the figures 32: 39: 49, and 72: 36: 12, obtained from pups tested with a mother continually exposed to Day 1 pups for 16 days, and pups tested with a mother housed with normally aged pups on Day 6. There is a significant difference between these ($\chi^2 = 37.94$, $p < 0.001$), suggesting that the Day 6 lactating mothers do emit more of an attractant than the mothers continually exposed to day old pups. Perhaps this comparison is not made because it does not fit in with the findings presented in another paper published the same year (Leon and Moltz, 1973), in which pheromone emission is postulated to occur only after Day 12, and to be on an all-or-none basis.

In later papers Leon and Moltz do not dismiss results with significance levels of more than 0.01 as being nonsignificant, and indeed, in Experiment 3 on quality of diet, Leon (1975) regards results with a probability greater than 0.05 as significant, in that he treats the data as indicative of a preference; "...the Diet B pups raised in the Diet A colony now show a preference, although not quite statistically significant, for the maternal pheromone of Diet A mothers." (p. 317).

Generally then, there appears to be some inconsistency over the selection of an α level and the consequent acceptance or rejection of the Null Hypothesis. This is rather overshadowed by the complete omission of any reference to the problem of selection of numbers for use in the Null Hypothesis comparison group in the χ^2 tests. It can be overcome by obtaining empirically derived sets of comparison numbers; otherwise, the researchers should at least state them, and justify their choice.

A letter from Leon provided the information that he dealt with the problem of non-choosers in the χ^2 test by halving their number and adding the value to the numbers of pups making goal box entries.

No information at all about the statistical method of evaluation appears in the publication "Endocrine Control of the Maternal Pheromone in the Postpartum Female Rat" (Leon and Moltz, 1973); in most of the subsequent publications the method of choice is the χ^2 test.

Leidahl and Moltz (1977; Figures 1 & 2) present results based on χ^2 analyses in which "p" values of < 0.001 and nonsignificance are given. It seems probable that in order to obtain these results these writers used the same method of comparison as Leon, although in the second group the number of non-choosers has risen to 30%. Not only does such a highproportion of non-choosers indicate that perhaps a different Null Hypothesis should be established, also, one wonders why the proprtion has risen from that reported in earlier studies.

In their presentation of results, Holinka and Carlson (1976) give significance levels obtained from using the χ^2 test; the comparison numbers they used are not stated in the publication.

Problems arising over the statistical treatment of results published in the literature can thus be summarised:

1. Details of which statistical tests are used for evaluation are frequently omitted.

2. α levels are not specified, and vary in an apparently arbitrary manner.

3. No control groups, providing empirically based sets of numbers, to use for comparison purposes are ever run.

4. There is no statement of the numbers used for comparisons with the χ^2 test, nor any indication as to how they were obtained.

III. Problems of interpretation and assertions which may be queried

Occasionally Leon and Moltz and their co-authors make claims which they consider are justified by the results they report. Critical inspection of their data shows that in some instances the conclusions are not supported. Indeed, this criticism applies to most of the empirical evidence which purports to indicate the existence of the maternal pheromone. In this section however, the major design errors are not discussed, as they have already been dealt with, but some less serious aspects are outlined.

In an early paper on the parameters of maternal pheromone, Leon and Moltz (1972), in Experiment 4, seek to discover whether it is emitted in an "all-or-none" fashion, or in gradually increasing and decreasing concentrations. Rat pups aged 16 days are given the choice in the olfactory discrimination apparatus of a 16 day lactating female, paired with females of 1, 10, 12, 14, 16, 21, 27 and 41 days post-partum. The investigators find that the pups do not prefer the 16 day old female to the ones of 14, 16 and 21 days post-partum, but that she is preferred to the remainder. These results are taken to support the idea that the pheromone is emitted in an all-or-none fashion, as had been suspected at the outset.

What the authors fail to consider, or establish, is what the results would have looked like if the pheromone had been emitted in gradually increasing concentrations. The pattern, in this case, would probably have been very similar to the one which they show in Figure 4 of their paper, yet the results are taken as supporting an all-or-none type of emission. A reproduction of Figure 4 from Leon and Moltz's paper is shown in Figure 5a of this thesis, and it can be compared with Figure 5b in which the pattern of results which might occur in an all-or-none type of emission is depicted.

It has already been pointed out in a previous section, that in another paper published in a later year, Moltz and Leon (1973) fail to draw attention to a comparison which can be made between two sets of data they present, and which indicate that an attractant might well be present at 6 days post-partum. As the model of pheromone production based on high prolactin levels and hyperphagia is advanced in later papers, only a gradually increasing level of pheromone emission would make sense, yet the earlier position about the all-or-nothing basis is never referred to or rescinded in later publications.

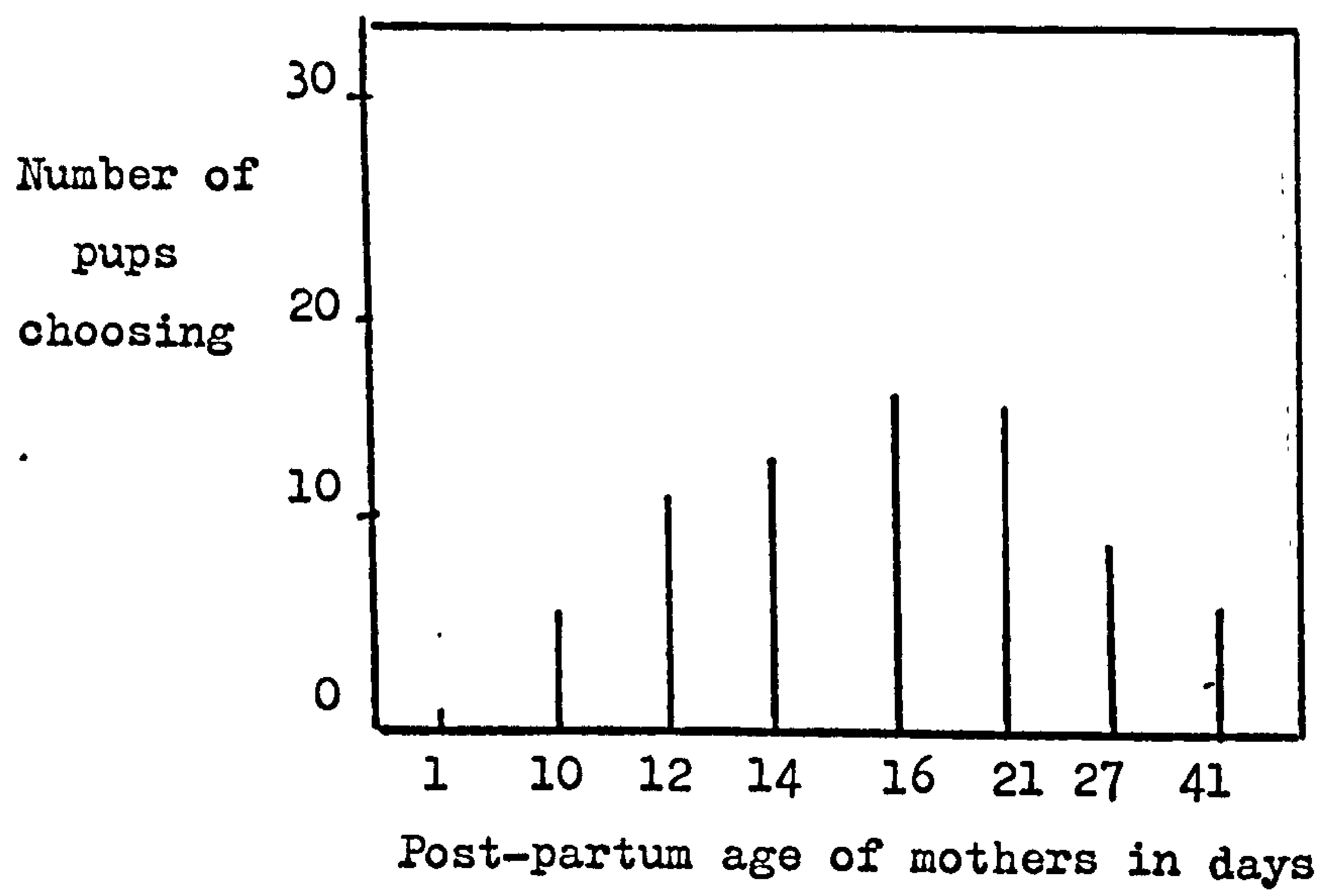


Figure 5a: Approach behaviour of 16 day young to females of differing lactational ages. (After Leon and Moltz, 1972; p. 686)

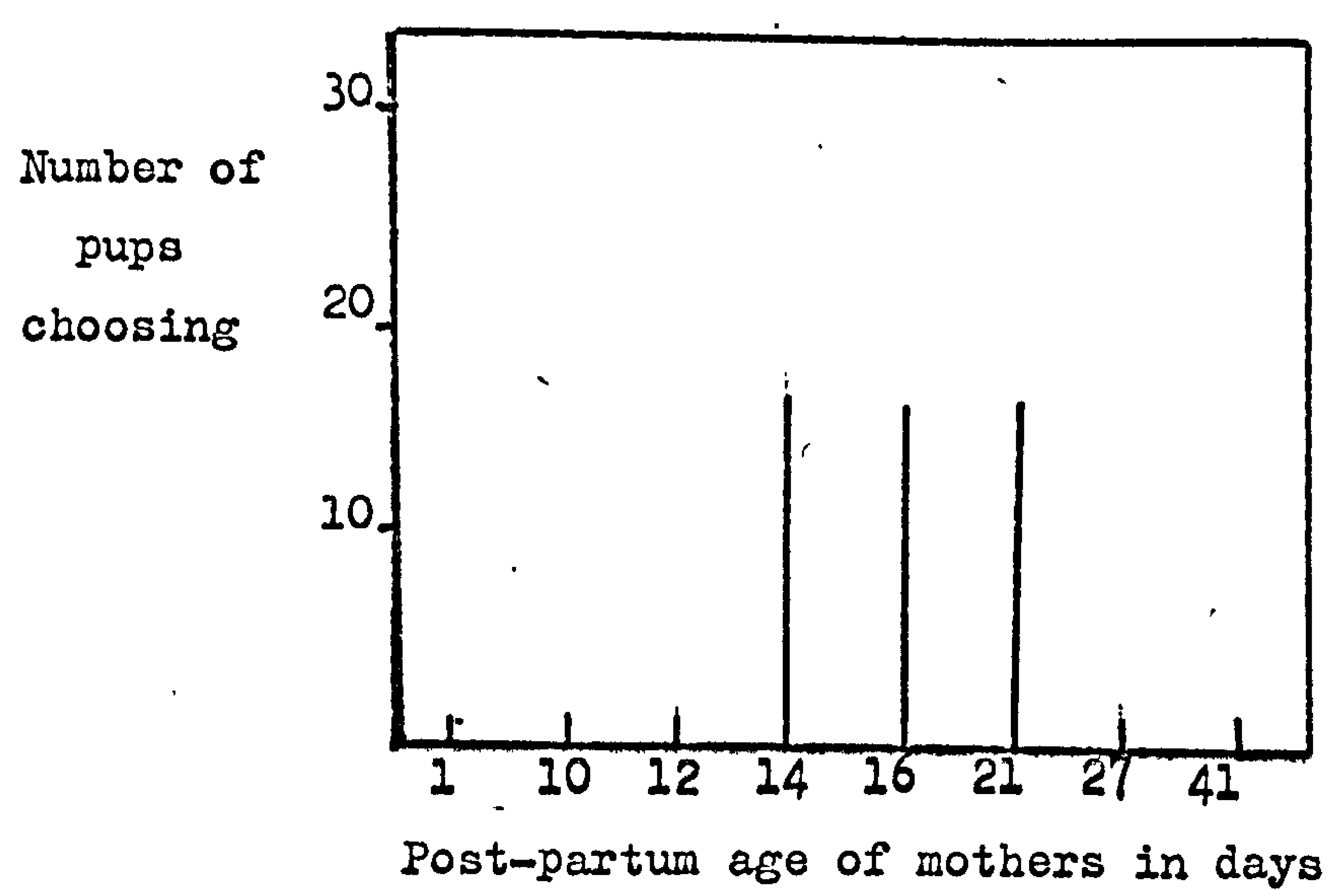


Figure 5b: Approach behaviour predicted in pheromone emission is on an all-or-none basis.

Throughout the publications on the maternal pheromone results from many comparisons are reported, often several comprising a single experiment. Leon's two papers "Maternal Pheromone" and "Dietary Control of Maternal Pheromone in the Lactating Rat" (1974, 1975) are very difficult to interpret because they present such a large number of comparisons involving many different variables. Instead of pursuing one topic thoroughly, and presenting a careful evaluation of it, Leon moves quickly through several aspects which involve numerous comparisons.

Occasionally it seems that crucial comparisons are not made. An example of this can be taken from the paper on dietary control (Leon, 1975). In the second part of the paper the reader is informed that if young are reared whilst constantly exposed to a particular kind of maternal excrement (or pheromone), then they will approach it preferentially at 16 days age. Two kinds of maternal excrement, one obtained from Diet A rats and one from Diet B rats are compared. However, what of the Diet S rats used in previous work? Diet S was first mentioned in the 1974 monograph; it is a diet which inhibits pheromone production, as its only carbohydrate is in sucrose form. A crucial comparison would have been to give Diet S rat pups the choice of their own mother's excrement versus a normal Purina fed mother's excrement, but this was not done.

Comparisons should be made more systematically and thoroughly; for instance after the manner advocated by Irwin (1961). Irwin provides a means of working out a continuum of attractability for two stimuli presented in odour preference tests; a minimum of three tests is needed for a single evaluation.

As it is, the American publications provide a welter of information which is almost impossible to disentangle. The writers have a consistent tendency however, to assure the reader that "clearly" such-and-such is the case, when the case is anything but clear.

In the explanation of pheromone emission which is put forward by Leon (1974, 1975) there is a heavy reliance on the role of gut flora. Some of Leon's assumptions and assertions can be questioned.

First, if a rat has a caecectomy, does that ensure that all the relevant bacteria are removed from the lower intestine?

Second; does the feeding of a different man-made diet to rats appreciably alter their gut flora - especially when they are kept together in the same housing rooms?

Third; if rats need caecotrophe and the bacteria it contains for adequate digestion of their own food, how is it that SPF animals and those reared in germ-free colonies do not appear to suffer any ill effects?

The Diet S rats used by Leon pose a few problems. He refers to them as "pheromone-free" (1975, p. 316); their anal excrement apparently does not attract young rat pups. Later in the same paper (p. 318) Leon states "The above data suggest that although all lactating females do not emit a single attractant, all lactating rats emit an attractant.", even though according to his results Diet S mothers do not appear to emit an attractant.

After the results presented on the effects of different diets, and the later imprinting model (Leon, Galef and Behse, 1977), it seems difficult to believe that the rats fed Diet S do not elicit approach behaviour, as the attractant properties of maternal excrement are to be regarded as a function of familiarity, rather than being due to a releaser-type action. There is no thorough investigation into why there is no evidence of an attractant present in the excrement of Diet S mothers. Is it that they produce no caecotrophe at all, or so little, that it is odour-free?

Not only is there a lack of careful work on Diet S rats, but it is likely that any work using the olfactory discrimination apparatus will not produce satisfactory answers. Gregory and Bishop (1975) expressed dissatisfaction with this technique, as well as with other relatively insensitive methods of behavioural bioassay. This opinion is reinforced by the difficulties which have been encountered during the experimental work carried out at Hull.

The most obvious problem, which became apparent after talking to Leon and Moltz in their respective laboratories, was that the maternal pheromone was not as pervasive a phenomenon as would appear from the literature. Leon had already mentioned in a letter that in some work carried out in Canada with hooded rats, the young behaved differently in the test apparatus from the albino rats. When details were published (Galef and Heiber, 1976), it transpired that to get the pups to enter goal boxes, they had to be handled daily for 5 days before testing. Moltz also mentioned strain differences which were apparent to researchers at Chicago (see Chapter XIII, Part II); and in fact, he was having some difficulty in obtaining the effect with the rats he was currently using. This was attributed to the effect of inbreeding, and he hoped to rectify it by obtaining more stock. It is difficult to reconcile this with a model of pheromone production in which prolactin has central importance. It would not seem likely that levels of this hormone, or its breakdown mechanisms, would vary in different groups of animals; a bacteriological explanation would appear to be more feasible.

IV. Post-1975 differences between the findings of Leon and Moltz

The ways that the work of Leon and Moltz diverged when Moltz started to examine the role of bile, whilst Leon became more involved in an imprinting type of explanation, was briefly mentioned when the relevant reports were summarised in Chapter XIII. Although in principle the two approaches are not incompatible, the divergence of their work has resulted in their presentation of certain conclusions which are difficult to reconcile.

Whilst Leon moves away from the position that the maternal pheromone is a single attractant, and explains the movement towards the odour as being due to a preference for the familiar, Moltz investigates the physiological mechanisms underlying what he believes to be a single substance. Through an inspection of the amounts eaten by concaveated maternally behaving nulliparous females, Leidahl and Moltz (1977, Experiment 4) demonstrate that elevated food intake is <u>not</u> a necessary condition for the emission of the maternal pheromone. This cannot easily be accomodated by Leon's model.

In an earlier paper, Leidahl and Moltz (1975) claimed that the pheromone was not emitted by male rats, even when they were successfully concaveated and showed maternal behaviour. It appeared to be this finding which led to research into the role of prolactin receptors in the liver, and the explanation that it is in the difference between male and female rats that the failure of emission in the male lies. However, Leon and Behse (1977) state that they have evidence, although it has not been published, that male rats do emit the maternal pheromone. This difference would also appear to be serious, in that Moltz's hepatic-prolactin model of pheromone emission hinges upon the inability of male rats to produce the attractant.

Finally, another difference between Leon and Moltz became evident after discussions with them. This was over the existence, nature, and importance of caecotrophe in the maternal pheromone explanation. This problem has already been described in Part II of the previous chapter.

Summary

Many of the criticisms made in the previous sections are serious. In particular, design faults present in almost all the published work on the maternal pheromone undermine what appeared to be a substantial body of evidence on its existence.

Problems of data analysis and interpretation are evident, and many of the assertions made appear to be of dubious value. There is a failure to pursue in a systematic manner any of the variables thought to play a role in pheromone production, and differences between Leon and Moltz, in their recent publications, cannot easily be reconciled.

Overall, these criticisms are a severe indictment of the North American work on the maternal pheromone.

CHAPTER XV

EXPERIMENTS ON OLFACTORY PREFERENCES USING SIMPLE APPARATUS

INTRODUCTION

There have been two major criticisms of the olfactory discrimination apparatus. The first is that it provides a complex rather than simple test procedure. Many variables, such as pups' experience of the apparatus, factors influencing open-field behaviour, and the relative attraction of the goal boxes with or without contents, must be considered, as they can obscure preferences which may exist.

Secondly, the apparatus is considered to be unsuitable for young pups, because it makes excessive demands upon them. Even if they are capable of certain discriminations, the fact that they have to move quite a distance to demonstrate this, made especially difficult if their pre-test maternal deprivation has left them with lowered body temperature, means that this test measure is inappropriate. Criticisms of the relatively crude measures obtained by means of behavioural bioassay have already been made. Ultimately, one would hope to see neurophysiological techniques used, giving a picture of the effects of various odours on the patterns of firing in the olfactory lobes of the brain. This kind of investigation is being undertaken in the study of visual processes (Hubel and Wiesel, 1965; Lettvin *et al.*, 1959).

Behavioural bioassay can provide good information about sensory processes, and has been used with great success by Nowell and his co-workers (Jones and Nowell, 1973c, 1975, 1977; Mugford and Nowell, 1971) in their investigations into the role of olfactory cues in aggressive behaviour in mice. The provisos are though, that the task does not require a response beyond the ability of the animals being tested, and that the response is a true reflection of the independent variable, not a function of any confounding variables, which can easily occur in the less controlled test situations which comprise behavioural bioassay.

In Experiment 5, a simple method for testing olfactory preference in young pups was described, when they had been tested in a plain box, the floor of which was covered with soiled and clean paper. In testing preferences for adult bodies, which was envisaged next, a method had to be developed where the bodies were invisible, yet the test situation was kept as simple as possible. Consequently, a piece of apparatus more complex than the simple box used in Experiment 5, yet simpler than the olfactory discrimination apparatus was needed. This was devised, and is described in detail in the next section. It will be referred to as the "Screen" apparatus. Two experiments are described in this chapter involving several comparisons made with this apparatus. The conclusions drawn are of a very limited nature however, and do little more than demonstrate the suitability of the apparatus for preference testing.

THE SCREEN APPARATUS AND TEST PROCEDURE

A litre plastic ice-cream container with opaque white sides, was cut in half, making two screens (15 x 14 cm), which would stand upright with support from the base and sides. At the bottom of each screen, a rectangular hole, measuring 4 x 2 cm was cut, positioned 1 cm away from the centre.

An animal which was to provide the olfactory stimulus for testing was anaesthetised using Immobilon, and then placed along the bottom of the screen, resting upon the inner base, and with the lower ventral surface exposed through the hole. The exposed area was held firmly in place by fastening the immobile animal closely to the screen with adhesive tape.

A large sheet of clean paper was placed on a table, and then the two screens, each with an immobile animal fastened in place, were put 15 cm apart, with the two exposed ventral surfaces directly facing each other. An aquarium, the sides of which had been covered with plain white paper, was then inverted and placed over the two screens, producing an airtight enclosure with non-transparent sides. The apparatus was placed immediately beneath a fluorescent light strip, so that it was illuminated evenly.

The screens, without the aquarium in place, but in the position used for testing, are shown in Plate 2.

When a pup was tested, the aquarium was raised slightly at one side, and the pup placed quickly into the centre of the rectangle made by its sides and the two screens. The pups behaviour over the next 5 minutes was observed by the experimenter, who could see through the transparent base of the aquarium.

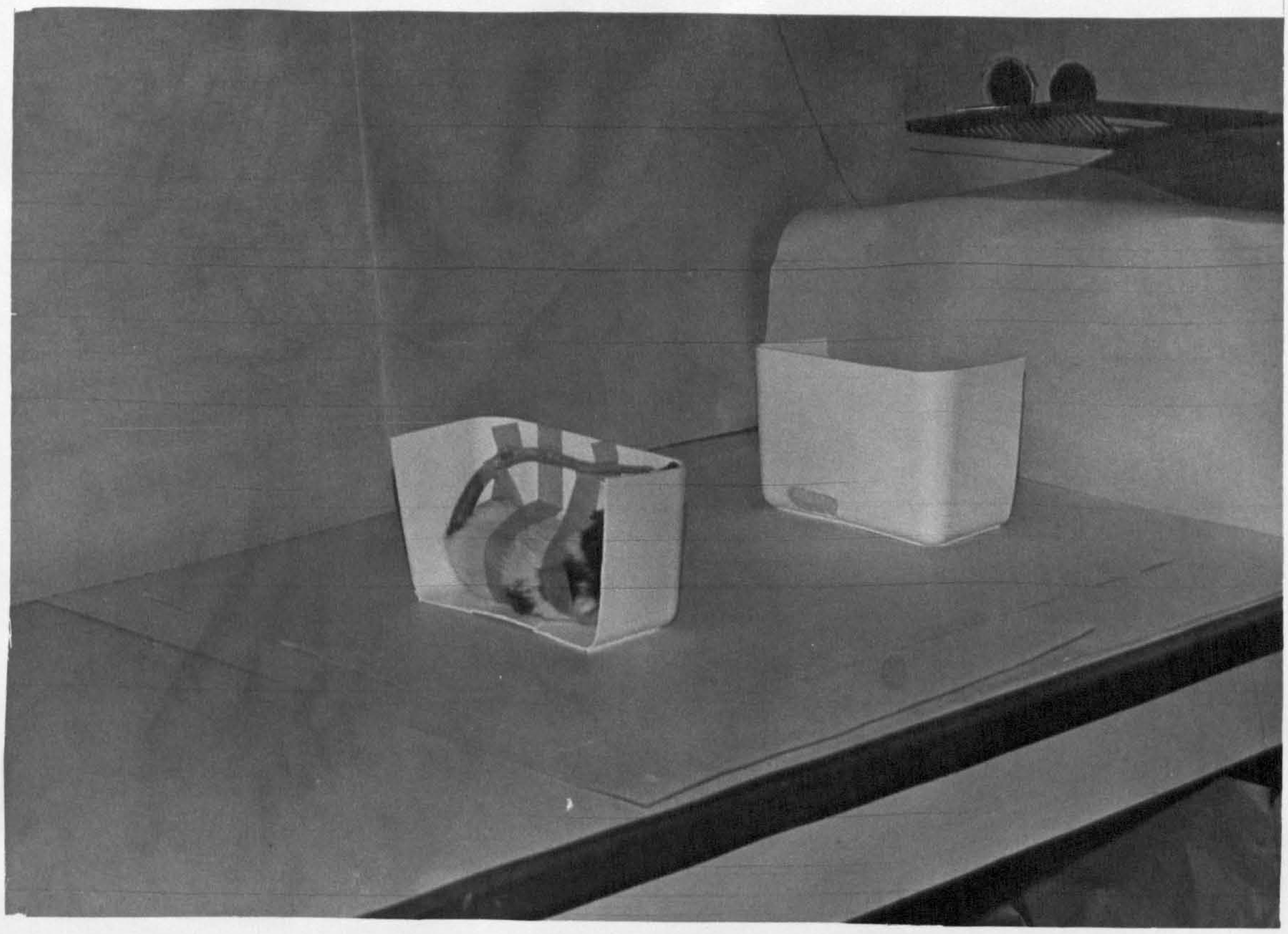
Typically, pups would move within the first few seconds, before becoming immobile. If they moved towards and touched one of the two exposed ventral surfaces this constituted a positive choice, and if they moved towards and remained against either the screens or sides of the aquarium, this was labelled "no choice". No pups displayed any behaviour which fell between these categories, reflecting their proclivity to move against walls, rather than remain in open spaces (Patrick and Laughlin, 1934).

It was necessary to use screens to provide four white sides to the test area, as it is known that young pups whose eyes are not open are able to discern and move towards dark shapes (Lee, 1975), such as the one made by an adult rat's body.

Each pup was tested once only in the apparatus; after half a litter had been tested the two stimulus animals were changed round. The walls of the aquarium and the screens were wiped after every trial.

When 10 day old pups were to be tested following a period of maternal deprivation, they were kept warm by placing the home cage in an incubator.

Plate 2



Anaesthetised rats held in position behind the screens. When pups are tested, the screens are enclosed by an inverted fish tank.

EXPERIMENT 16: PREFERENCES SHOWN BY 10 DAY OLD RAT PUPS

Purpose

This experiment was regarded as a preliminary investigation into preferences shown by 10 day old rat pups for immobile adult rat bodies. A number of comparisons were made, using lactating and non-lactating females, and male rats.

In view of the results obtained in previous experiments, no experimental predictions were made; the test pups' behaviour would be compared with that of a control group run in the empty apparatus.

Subjects

205 PVG/C rat pups, aged 10 days, from litters born to nulliparous female rats, mated at 100 ± 10 days, were used. Litters were culled to 6 on Day 0; a few litters comprised 5 pups because of a death after culling.

Stimulus animals used were the pups' own mothers, 4 nulliparous females and 6 male rats of the same age as the mothers.

The 4 nulliparous females were kept in the same colony room as the mothers and their litters, along with 3 of the 6 males. The other 3 males were housed in another colony room well away from the experimental animals.

Maintenance was as described in Part I of Chapter III; the adult rats were fed the Oxoid diet.

Apparatus

The Screen apparatus was to be used for testing pups' olfactory based preferences.

Procedure

Following the procedure already outlined, the 10 day old pups were to be tested either without any maternal deprivation, or following a 3 hour period of maternal deprivation.

In all the comparisons to be made, one of the stimulus animals was the pups' own mother. The others were:-

1. Nulliparous female (no pre-test deprivation)
2. Nulliparous female (3 hours pre-test deprivation)
3. Male housed in colony room (no pre-test deprivation)
4. Male housed in colony room (3 hours pre-test deprivation)
5. Male housed in different room (no pre-test deprivation)

A group of pups tested in the empty apparatus would serve as a control.

Results

The pups' preferences are shown in Table 22. The control animals all moved away from the centre of the apparatus and without exception ended up in one of the 4 corners. As 14 chose one side of the apparatus, and 16 the other, this showed that there was no biasing factor present in the apparatus or test procedure.

Table 22

Preferences shown by 10 day old rat pups for immobile bodies, using the screen apparatus

GROUP	PRE-TEST DEPRIVATION (hours)	STIMULUS ANIMAL	STIMULUS ANIMAL	NO CHOICE	N	p
		Mother	Virgin			
1.	0	32	32	0	64	ns
		Mother	Virgin			
2.	3	16	12	0	28	ns
		Mother	Male from same room			
3.	0	16	8	0	24	ns
		Mother	Male from same room			
4.	3	11	2	16	29	0.01
		Mother	Male from diff room			
5.	0	27	3	0	30	0.001
		No animal	No animal			
Control	0	0	0	30	30	

Inspection of the data show that all the groups tested with animals present behind the screens differ from the control group. As in all of the groups but one (Group 4), the pups chose to approach either of the animals, binomial tests were carried out on the data. The probability levels obtained are given in the final column of the table. On the data from Group 4, the Kolmogorov-Smirnov test was used for the comparison with the control group, as the expected cell frequencies were too small for the χ^2 test to be used.

Discussion

The results obtained when animals are placed behind screens, and the pups which are tested only have to make a simple movement, are clear-cut and easy to interpret.

Pups aged 10 days failed to distinguish their own mother from a non-lactating female. In Group 1, pups would approach either of the exposed ventral surfaces and start to nuzzle into the fur, even though they had not been deprived of maternal attention before the test. The pups that went to the mother would start to suckle keenly if they found a nipple. It had been thought, after this group had been run, that perhaps the 3 hour period of deprivation given to the pups in the second group would encourage them to make a discrimination between the two females, but the results indicated that there was still no preference for the mother. That the pups were able to make a choice, and had not become too cold in the pre-test period, was evidenced by the fact that every single pup approached one of the ventral surfaces, rather than failing to move.

In Group 3, one-third of the pups tested chose the male animal, and when the comparison was repeated (Group 4), after a pre-test deprivation period of 3 hours, that proprtion fell, and there was evidence of a preference for the mother ($p = < 0.01$).

As the male rats used in comparisons 3 and 4 had been housed in the same room as the test pups, a final group, using males from another colony room was run. It was considered that these animals may have a different, and unfamiliar odour. The results differed from both the control group ($p = < 0.001$, Kolmogorov-Smirnov test) and from Group 4 ($\chi^2 = 15.234$; df = 1; $p = < 0.001$; using pooled cells and Yates' correction), but not from Group 3 ($\chi^2 = 3.360$; df = 1, using Yates' correction). Further experimental comparisons would have to be made to determine the extent of the influence of pre-test deprivation; also, the question of whether the pups were approaching the mother because of an attractant, or because they were avoiding the male rat.

An interesting qualitative difference in the pups' behaviour whilst being tested in this apparatus was observed. This was that when pups approached the ventral surface of any female rat, whether a mother or a non-lactating female, they nuzzled vigourously into the fur. When pups approached a male however, although they remained in close proximity, they were relatively immobile and rarely nuzzled into the fur.

Taking the results as a whole, it was concluded that pups could not distinguish their mother from a non-lactating female rat, and that their behaviour towards males was not very different when the odour of the males was familiar to them. However, pups did not approach males which had a strange odour, and in this case preferred the mother. Even though the pups had approached males with a familiar odour, that they could distinguish them from females was evidenced by the fact that they failed to show the vigourous nuzzling movements which they exhibited when they were close to the females. Perhaps this difference is due to the skin lipids secreted by the females; these have been studied by Hofer et al., (1976) in their research into the suckling response by young pups, and are mentioned by Nicolaides (1974) in connection with female animals.

EXPERIMENT 17: PREFERENCES SHOWN BY 18 DAY OLD RAT PUPS

Introduction

Although Holinka and Carlson claimed that the maternal pheromone was emitted by mothers as early as 8 days post-partum, Leon and Moltz give 14 days as the age of onset. It could be that an attractant is emitted by the Hull PVG/C rats, starting after 10 days post-partum.

Purpose

The aim of this experiment was to determine whether 18 day old rat pups would, after 3 hours maternal deprivation approach their mother's body when it was compared with that of a non-lactating female. This comparison was carried out using two strains of rat pups, the hooded PVG/C and Wistars. The PVG/C pups would also be presented with the choice of their own mother versus a male, and two male rats, both of which had previously been kept in the same colony room.

Subjects

The following PVG/C rats were used: 15 lactating female animals which had been mated at 100 ± 10 days, and their 90 pups aged 18 days at the time of testing; 4 nulliparous female and 6 male rats of the same age as the mothers.

24 Wistar pups were tested at the same age. These were litters from 4 female animals which had been mated at 100 ± 10 days. These adult females were also used in the comparisons made.

All the experimental animals were housed in one room.

Litters were culled to 6 on Day 0, all animals were fed the Oxoid diet, and maintenance was as described in Part I of Chapter III.

Apparatus

The Screen apparatus was to be used to test the pups' preferences.

Procedure

Using the standard test procedure with the screen apparatus, 4 groups were tested and given the following comparisons:

1. PVG/C pups: mother versus nulliparous female
2. Wistar pups: mother versus nulliparous female
3. PVG/C pups: mother versus male housed in same room.
4. PVG/C pups: two males, both housed in the same room as the pups.

A control group was also set up, in which the 18 day rat pups were to be tested in the empty apparatus.

All pups underwent 3 hours maternal deprivation before testing.

Results

The choices made by the pups are shown in Table 23.

Table 23

Preferences shown by 18 day old rat pups for immobile bodies, using the screen apparatus

GROUP	STRAIN	STIMULUS ANIMAL	STIMULUS ANIMAL	NO CHOICE	N
1.	PVG/C	Mother 11	Virgin 10	3	24
2.	Wistar	Mother 11	Virgin 11	2	24
3.	PVG/C	Mother 5	Male from same room 9	17	31
4.	PVG/C	Male from same room 2	Male from same room 7	26	35
Control	PVG/C	-	-	-	-

Although testing started with the control group, it was abandoned after one litter, as the pups explored the apparatus during the time allowed (5 minutes). They climbed through both holes cut in the screens and constantly moved round, making categorisation of responses impossible.

Statistical comparisons carried out between the various groups will be described in the next section, where the behaviour of these rats is discussed.

Discussion

Inspection of the data from Groups 1 and 2 reveals no evidence that the maternal animal was preferred to another female - either in the PVG/C or Wistar rat pups which were tested. There appears to be no difference between the two strains, either.

When the PVG/C pups mother was opposed to a male which had been housed in the same room, a higher proportion now failed to make any choice (χ^2 test carried out between the results from Group 1 and Group 3 gives a value of 10.55 for χ^2; df = 2; p = <0.01), and there is a slight (but nonsignificant) preference for the male.

The number of non-choosers rose even more when males only were placed behind the screens.

The conclusion from this experiment is not very different from that reached in the previous one. There is no evidence that 18 day PVG/C and Wistar rat pups are attracted to their mother in preference to a non-lactating female.

Conclusions from Experiments 16 and 17

Although PVG/C rat pups as young as 10 days were able to discriminate a male from a female rat on the basis of odour, there was no evidence that the maternal rats, either at 10 or 18 days post-partum, emitted an attractant. When the young approached the ventral surface of any female, either nulliparous or lactating, they exhibited vigourous nuzzling movements, suggesting that some stimulus characteristics initiated a suckling response. This stimulus is apparently absent in the male.

It could be argued that the maternal pheromone is present in anal excrement, and even if emitted by the animals used in these experiments, it would not be present in a sufficient quantity, on the fur of the animals, to attract the young. If this is the case, then the approach behaviour obtained here lends weight to the assertion made by the present writer that the positive results obtained with live animals in the American studies may have been partly due to olfactory cues from the body, not just the excrement. It would be quite easy to devise a piece of apparatus similar to the Screen apparatus, in which pups' approach responses to excrement samples could be assessed.

It is of interest to compare these experimental results with those obtained in Experiment 5, in which 12 day rat pups had been tested in a simple box, and their preference for odour present on the floor covering assessed. Here the 12 day pups had shown a preference for the maternal odour when it was present on one half of the box floor, as opposed to choosing the odour-free side. The odour derived from the mothers must have been a mixture of urine, traces from faecal matter and any other bodily secretions produced. It would be possible to use the simple arena to determine whether pups preferred a floor covering previously soiled by the mother to one soiled by a non-lactating female. On the basis of the results obtained in Experiments 16 and 17, it would be predicted that the pups would not display a preference; on the basis of the earlier work, in which a consistent slight preference for maternal excrement was obtained, the prediction would be for a preference for the maternal side.

It must be emphasised that the experiments reported in this chapter are not meant to comprise a final evaluation of the olfactory preferences and abilities of pre-weanling rat pups. The Screen method demonstrated that pups as young as 10 days are influenced by olfactory cues. Whether the source of these is in the adult animals' skin secretions and fur, excrement or urine remains a matter for further investigation.

CHAPTER XVI

CONCLUSION

Experimental work on olfactory preferences in rat pups

The shortcomings of the published work on the maternal pheromone have already been described and discussed in Chapter XIV. The conclusion reached was that faults in experimental design and analysis meant that most of the findings should be regarded as unsound.

It would not be denied, however, that many of the rat pups tested by Leon and Moltz and their colleagues are attracted towards a maternal odour under some circumstances; what is questionable is that the attractant has the compelling powers of a pheromone, and that it is contained in the type of anal excrement known as caecotrophe. In much of the work reported, where live animals were used in the goal boxes, odours emanating from the body, rather than from the excrement might have elicited approach responses from the pups. Nyakas and Endrőczi (1970), Holinka and Carlson (1976), and Galef and Heiber (1976) suggest that maternal urine has attractant properties. This was found in Experiments 6, 7 and 10 of this study, when preferences for urine and urine-stained home-cage bedding were noted.

The failure of maternal excrement to attract rat pups, found not only in this study, but also by Kendrick (1975), Galef and Heiber (1976) and Moltz (personal communication), indicates that the maternal pheromone is not a general characteristic of the laboratory rat, as Holinka and Carlson (1976) claim. The fact that the attractant is not common to all members of the species *Rattus norvegicus* also means that strictly speaking, it cannot be called a pheromone.

Whilst it may be the case that attractants do exist, their powers and chemical compositions are as yet unknown. Certainly young rat pups can make olfactory discriminations. This has been amply demonstrated in many of the experiments reported in this study, but particularly in Experiments 5, 16 and 17. The ability of these pups to distinguish lactating from non-lactating female rats is open to question however. The results of Experiment 8 and the comparisons using the Screen apparatus indicate that female rats in general are attractive to pre-weanling rat pups. Perhaps the odours these animals give off causes an increase in the pups' general arousal level, and this may then bring about searching behaviour, nuzzling and suckling responses.

Under certain conditions excrement and urine samples appear to have slightly aversive qualities (Experiments 4, 9 and 11), but this effect was not a consistent one, and did not appear to vary as a function of method of excrement collection, as was hypothesised at one stage. The problem of stress induced by isolating animals in metabolic cages and assessing the effects of various secretions upon conspecifics is an exceedingly complex one.

Strength of odour would seem to be a variable worthy of consideration. It is mentioned by Leon (1974, Experiment 2), and examined specifically by Galef and Heiber (1976). How much this varies as a consequence of the moisture content of faecal matter, and whether it is a reflection of the actual chemical components of the products would be an exceedingly difficult problem to tease out. Indeed, a satisfactory solution to the problem of separating urine from anal excrement, without sacrificing the donor animal, has yet to be devised.

Whilst the experimental work reported in this study was in progress, there was for quite a long time, a belief on the part of the experimenter, that the female rats were producing the maternal pheromone, or were capable of producing it, given the right conditions. Almost every time a new series of comparisons started, it was expected that on this occasion the "overwhelming preference" shown by rat pups on the other side of the Atlantic would appear. Every time, disappointment followed, as the rats consistently failed to be attracted towards the maternal excrement odour. It was not until 15 experiments had been completed, and the visit to the North American laboratories had taken place, that it began to be accepted that the rats tested in Hull had not been emitting the maternal pheromone.

The problem of the analysis of results, when a large proportion of the rat pups failed to enter a goal box in the olfactory discrimination apparatus, and the conclusion that generally their behaviour was not much different from that exhibited by pups placed in the empty apparatus, was often felt to be obscuring a preference for the maternal odour. This preference was indicated by the slightly higher number of goal box entries for the maternal, as opposed to the non-maternal stimulus. It was only after all the experiments had been run that this tendency could be examined and evaluated.

Of the 21 comparisons reported in which the olfactory discrimination apparatus was used, pups preferred the maternal stimulus in 16. Although only 3 of these 16 showed a significant difference when the binomial test was carried out on the goal box entry numbers alone, taking all 21, the two-tailed probability of obtaining a division into 16 and 5 is 0.026. In other words, it would appear that the 17/18 day old rat pups tested exhibited, over the course of the experimental work, a preference for the maternally derived stimulus.

The nature of the substrate or substrates contained in the excrement (or urine which might have contaminated the samples) remains a matter for further investigation. As has already been indicated, there are several methodological problems to be overcome before valid experimental work in this area can take place.

The consistent failure to replicate published findings on the maternal pheromone revealed the difficulties inherent in making a negative statement about an apparently well established phenomenon.

Not only does a great deal of work have to be carried out before one can have any confidence in the negative findings, but the proponents of an established phenomenon are always able to point to some variable in the replication which differs, and attribute the failure to that.

It cannot be denied that small details of routine maintenance and housing exert an influence on the behaviour of laboratory animals, and as Meier (1968) points out, the number of such variables is constantly increasing. However, not only are replications in animal work difficult, where the organisms studied exhibit constant change and sensitivity to an enormous number of variables, but they are also considered to be unsuitable material for publication.

Meier states:

> Whatever the cause or rationalization, the trend of scientific protocol that holds in disdain the replication of published researches must be reversed. Under present conditions, the level of achievement in contemporary science is obscured and its future development retarded.
>
> (Meier, 1968; p. 78)

He feels that in failing to report both positive and negative outcomes of replications, scientists are in danger of missing making new findings.

The maternal pheromone may be a case in point. As it would appear that under some conditions lactating females do emit olfactory cues with limited powers of attraction for pre-weanling pups, it is regrettable that more research effort is not directed towards a careful and valid evaluation of this phenomenon, rather than the investigation of complex aspects of an effect now known to be of limited generality.

Recent use of the term "pheromone"

Definitions of "pheromone" were given in Chapter I, and the trend towards using the term in an increasingly vague, and explanatory fashion indicated. Kennedy (1969) is also critical of the way in which the term "attractant" is used in a blanket fashion. As "attractant" is virtually synonymous with "pheromone", the comments he makes on attractants are of some relevance.

In talking of insect behaviour, he suggests that a female sex pheromone may at first merely activate a male, and cause a change in its central nervous system, so that the insect is responsive to different kinds of stimuli than in the "non-aroused" state. Consequently, differential responses may be shown to such environmental features as air-flow or light. His argument is that the concept of an attractant merely releasing a particular response is too simplistic, even in explaining insect behaviour. Simple explanations are even more unlikely when the behaviour of mammals is considered.

It has been recognised for some time that comparisons between insect and mammalian pheromones must approximate the relationship between the capacities of their different nervous systems. Mammalian behaviour is so adaptable and experientially determined that the inflexibility implied by the term "pheromone" is completely out of place. It would seem preferable to discard the term, and replace it with something like "organic chemical", which lacks the connotation of behavioural rigidity.

Some work on olfaction has been carried out on primates, including man. Michael and Keverne (1968) demonstrated that female rhesus monkeys produce a powerful sex attractant which is composed of fatty acids modified by bacteria present in the vaginal secretions. More recent work has shown that the same short-chain fatty acids are produced in other species of monkeys, in baboons, and humans. However, the problems which arise when attempting to apply the pheromone concept, with its restrictive implications, are highlighted when olfaction is studied by default.

If male monkeys, whose sense of smell has been temporarily blocked, were exposed to sexually receptive females with which they were not previously aquainted, they showed no response. However, if they were exposed to females with which they had previously mated, then even without their sense of smell they would try to gain access to these sexually receptive partners (Stoddart, 1976; Epple, 1974) If rats are rendered anosmic, they are able to eat, mate, give birth to and rear young with almost their normal degree of success. It seems that their behaviour is not dependent upon olfaction - or for that matter, any other single sense (Herrenkohl and Rosenberg, 1972; Barnett, 1975).

Possibly rats are similar to man in this respect; they have the capacity to develop and use many sense modalities in the event of one being damaged or impaired. This is in contrast to mice, whose behaviour starts to disintegrate when they are deprived of their sense of smell (Bronson, 1974; Rowe and Edwards, 1972; Gandelman, 1971)

In considering relatively complex mammals then, the term "pheromone", for olfactory stimuli is not a useful one to employ, and may be misleading. Not only do such stimuli fail to elicit a specific reaction if the context and animal's experience render it inappropriate, but in addition, the loss of sense of smell may make remarkably little difference to the behaviour observed.

However, once a phenomenon has been labelled, it often comes to stay, regardless of whether or not it is useful, and sometimes even when its very existence is open to doubt. This seems to be the case with the term "pheromone", as it is now being applied indiscriminately to almost any olfactory cue.

Given below, are some examples of the type of statements appearing in the press.

"Triggers behind a maternal pheromone"

Science News, 111, 15, p. 233, 1977

This is a statement which does not encourage the reader to approach the existence of the maternal pheromone with a questionning attitude.

"The sweet smells of sex"

Psychology Today, 3, 6, p. 18-23, 1977

contrasting with

"Not so sweet but, oh, so sexy"

Hull Daily Mail, 31.10.75., p. 19.

The articles following these last two headlines are of interest in that they report recent pheromone research findings in layman's terms. Whilst neither employ the word "pheromone", both communicate the more spectacular connotations of the concept. The authors apparently differ in their opinions as to whether the smells underlying our sexual behaviour are pleasant or unpleasant. Odourless substances, or the possibility of subliminal perception are not mentioned.

In New Scientist Burton informs the reader:

> Human females (and males) have evolved the peculiar habit of carefully erasing their own natural pheromones which have presumably evolved to be stimulating to members of the opposite sex.
>
> New Scientist, 75, 1063,

This writer goes on to speculate that the increasing use of perfumes may lead to a rise in the incidence of homosexuality. Whilst the question itself is perfectly valid, what is regrettable is the way that such terms as "pheromone" and "homosexuality" are being used as if they are well-established, well-understood phenomena.

"Pheromone" is rapidly sinking to the same depths as that other overworked, misunderstood word, "instinct". Both rely upon too many unproven assumptions and both provide facile pseudo-explanations for phenomena which are likely to be multivariate.

References to pheromones in connection with human behaviour are becoming more frequent; short reviews are provided by Comfort (1974) and Stoddart (1976). The last example to be given here comes from an American magazine.

> "PHEROMONES........Yes, there are odors that turn him on, and they don't cost a cent!"
>
> Ultra, 1.2.1977

This verges on science fiction. Whilst it is not to be denied that olfaction may be of importance in human behaviour, and perhaps is underestimated, the implication that we operate in a simple "lock and key" fashion, as suggested by the users of the term "pheromone", in the examples given above, is making a mockery of the complex subtlety of human behaviour.

Maternal pheromone in the rat

Consideration of the term and concept "pheromone", would indicate that pheromonal-type agents are the exception rather than rule in rats; mammals which display a high degree of complexity.

Although a "maternal pheromone", i.e. a substance which elicits approach responses from young animals by their mothers, is a plausible, and somewhat appealing concept, the experimental evidence reported in the published literature and in this study, would suggest that maternal odour or odours may have only limited powers of attraction for the offspring. Strictly speaking, these odours should not be referred to as pheromones.

To date, there appears to be no evidence that an agent with compulsive powers of attraction - the maternal pheromone - exists.

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APPENDICES

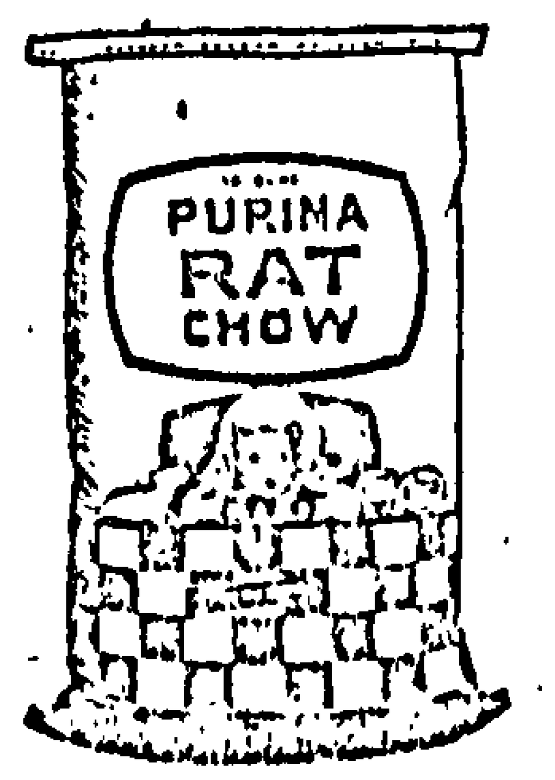
APPENDIX I

The constituents of the animal diets used.

APPENDIX I

PURINA RAT CHOW

CODE: 5012



Protein, min. 22.0
Fat, min. 4.0
Fiber, max. 5.0

INGREDIENTS:

Ground extruded yellow corn, soybean meal, fish meal, wheat germ meal, dried beet pulp, ground oat groats, wheat middlings, dehydrated alfalfa meal, cane molasses, soybean oil, dried brewers' yeast, methionine hydroxy analogue calcium, vitamin B_{12} supplement, calcium pantothenate, choline chloride, riboflavin supplement, thiamin, niacin, vitamin A supplement, D activated animal sterol, vitamin E supplement, calcium carbonate, dicalcium phosphate, calcium iodate, salt, zinc oxide, manganous oxide, iron oxide, cobalt carbonate, copper oxide.

FEEDING DIRECTIONS:

Rat Chow is in Checker form. It is a complete life-cycle ration for rats, mice and hamsters. Where a high energy diet is desired for mice and hamsters during gestation and lactation use Mouse Chow.

Provide feeders large enough to hold two to three days' supply of Rat Chow at one time. Arrange feeders so that animals cannot contaminate feed with feces. Keep plenty of clean fresh water available to the animals at all times.

APPROXIMATE CHEMICAL COMPOSITION*

NUTRIENTS**							
PROTEIN %	22.8	FAT %	4.5	Fluorine, ppm	65.0	Niacin, ppm	60.0
Arginine %	1.42	FIBER %	3.8	Iron, ppm	197.0	Pantothenic Acid, ppm	12.5
Cystine %	.35	TDN %	77	Zinc, ppm	30.3	Choline, ppm X100	19.0
Glycine %	1.12	NFE (by difference) %	52.9	Manganese, ppm	54.4	Folic Acid, ppm	1.7
Histidine %	.58	Gross Energy, KCal/gm	4.16	Copper, ppm	15.1	Pyridoxine, ppm	4.5
Isoleucine %	1.22	ASH %	6.0	Cobalt, ppm	.37	Biotin, ppm	.30
Leucine %	1.85	Calcium %	1.01	Iodine, ppm	1.17	B-12, mcg/lb.	9.0
Lysine %	1.36	Phosphorus %	.74	VITAMINS		Vitamin A, IU/gm	12.0
Methionine %	.43	Potassium %	1.08	Carotene, ppm	5.6	Vitamin D, IU/gm	3.3
Phenylalanine %	1.07	Magnesium %	.21	Menadione (added), ppm	–	Alpha-tocopherol, IU/lb.	17.0
Threonine %	.89	Sodium %	.36	Thiamin, ppm	10.9	Ascorbic Acid, mg/gm	–
Tryptophan %	.27	Chlorine %	.43	Riboflavin, ppm	4.5		
Valine	1.17						

*Based on latest ingredient analysis information. Since nutrient composition of natural ingredients varies, analysis will differ accordingly.
**Nutrients expressed as per cent of ration except where otherwise indicated. Moisture content though variable is assumed to be 10.0% for the purposes of calculations.

PURINA RAT CHOW

APPENDIX I (Cont'd)

Calculated Analysis

	P.R.D.	P.M.D.
Crude Oil	2.78%	2.78%
Crude Protein	19.79%	19.79%
Crude Fibre	5.37%	5.37%
Calcium (as Ca)	0.72%	0.72%
Phosphorus (as P)	0.71%	0.71%
Salt (as Na Cl)	1.03%	0.53%
Metabolisable Energy	2570 kcal/kg.	2580 kcal/kg

Trace Elements added

		P.R.D.	P.M.D.
Manganese	ppm	25	25
Copper	ppm	7	7
Cobalt	ppm	0.4	0.4
Iron	ppm	30	30
Iodine	ppm	1.3	1.3
Magnesium	ppm	102	102

Amino Acids (as percentage of feed)

	P.R.D.	P.M.D.
Threonine	0.77	0.78
Glycine	0.97	0.97
Valine	1.04	1.04
Isoleucine	0.58	0.83
Leucine	1.58	1.58
Tyrosine	0.76	0.76
Phenylalanine	0.94	0.94
Histidine	0.51	0.51
Arginine	1.25	1.25
Tryptophan	0.25	0.25
Methionine	0.36	0.36
Cystine	0.27	0.27
Lysine	1.07	1.07

Vitamins added per kg

	P.R.D.	P.M.D.
Vitamin A	8,000 iu	8,000 iu
D_3	1,000 iu	1,000 iu
B_2	8 mg	8 mg
Nicotinic Acid	20 mg	20 mg
Pantothenic Acid	4 mg	4 mg
Vitamin B_{12}	12 μg	12 μg
E	25 iu	25 iu
K	10 mg	10 mg
Folic Acid	6 mg	6 mg
Choline Chloride	200 mg	200 mg
Vitamin B_1	2 mg	2 mg

[illegible],
Christopher Hill Group Ltd.,
P.O. Box 6,
Agrarian House,
Castle St.,
Poole, Dorset.

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APPENDIX I (Cont'd)

Labsure Animal Foods

RHM

THE CHRISTOPHER HILL GROUP LIMITED
P.O. BOX 6, AGRARIAN HOUSE, CASTLE STREET, POOLE, DORSET, BH15 1HL (REGISTERED OFFICE)
TELEPHONE: POOLE (02013) 70561. TELEX: 41135

Please find enclosed an up to date brochure for your records., and listed below please find the constituents in CRM as requested:

Barley
Wheat
Maize
Oatfeed
Soya
Labmin/Labvits
White Fish Meal
Lysine,
Choline Chloride
Salt
Chalk
Di Calcium Phosphate.

We hope the above information will be of value to you and assure you of our best attention at all times.

Yours sincerely,
LABSURE ANIMAL FOODS

C. M. Foote

C. M. Foote (Mrs)
Secretary to J. E. Turnbull

Registered in England No. 310061

APPENDIX I (Cont'd)

BRADSHAWS

E. B. BRADSHAW AND SONS LIMITED
Bell Mills · Driffield · Yorkshire YO25 7XL
Telephone Driffield 3163 (4 lines) Telegrams Bradshaws, Driffield

FLOUR MILLERS · ANIMAL FOOD MAKERS · AGRICULTURAL MERCHANTS

AEJB/SW

15th March, 1973

The Department of Zoology,
The University,
HULL,
HU6 7RX

For the attention of Mr.K.R.Hoggarth

Dear Sir,

We have finalised our research into the proposed Bradshaw Rat and Mouse Breeder Diet and are pleased to offer to you as follows:-

Rat and Mouse Breeder Diet in 4 ton lots £83.00 per ton delivered Aldbrough. The diet would be packed in 56 lb. plain paper bags. The price would be subject to 2% discount for payment 28 days.

The calculated analysis of this diet is as follows:

Oil	3.25
Protein	21.10
Fibre	3.18
Calcium	1.32
Phosphorus	1.03
Salt	0.80
Magnesium	0.22
Potassium	0.91
Linoleic acid	1.03
Lysine	1.29
M+C	0.74
Arginine	1.17
Histidine	0.49
Isoleucine	1.10
Leucine	1.67
Phenylalanine	0.98
Threonine	0.78
Tryptophan	0.28
Valine	1.09
Gross Energy	3875 Kcals/Kg
Met. Energy	3487 " "

The following comments on the calculated data are apposite:-

1) The oil, protein, fibre, calcium, phosphorus, lysine and M+C data are all comparable with the competing product. They are at levels which should meet the requirements of the rat for breeding purposes with the possible exception of M+C for lactation. In this latter case the recommended value is 1% while for gestation 0.7% is suggested. We would seriously doubt whether many colony diets achieve the higher value.

cont....

APPENDIX I (Cont'd)

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ADJS/SW 15th March, 1973.

cont...

2) The gross energy is calculated from the analysis of the diet and will be seen to be lower than that of the competing product. However, applying the same principle to the competing product and assuming the ash content to be 8% versus 8.38% in your mix we calculate a value of 3892 Kcals/Kg. Thus the two diets are comparable. The metabolisable energy value is usually taken to be 90% of the gross energy - this convention has been adopted.

3) The level of linoleic acid is calculated to be well in excess of minimum requirements and above the competition. S. Fat has been assumed to contain equal proportions of PK, groundnut and soya oil in its ether extract.

4) Magnesium and potassium are well above minimum and above the competing product.

5) The amino acids are calculated to be present in adequate amounts with the possible exception of Histidine. This latter is adequate for growth, but for gestation and lactation it could be increased to 0.54%. To do so would probably necessitate an increase in the protein content - particularly as animal protein.

6) We have not made calculations on glycine, aspartic acid, glutamic acid or proline because these are non-essential amino acids. Neither is tyrosine calculated because this can subsitute for phenylalanine up to ⅓ in much the same way as cystine for methionine. The level of phenylalanine is adequate on its own but cannot be satisfactorily utilised in the absence of tyrosine. However the latter will be present in sufficient quantity.

7) Trace element additions are as follows:

Iron	100	g/ton
Copper	10	"
Manganese	50	"
Cobalt	1	"
Zinc	20	"
Iodine	2	"

8) Vitamin additions are as follows:

Vitamin A	12	m.i.u/ton
" B_1	6	g/ton
" B_2	6	g/ton
" B_6	2	g/ton
" B_{12}	10	mg/ton
" E	50	g/ton
" K*	5	"
Folic acid**	0.75	"
Nicotinic acid	30	"
Pantothenic acid	10	"

cont...

APPENDIX I (Cont'd)

- 3 -

15th March, 1973.

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Choline chloride	500	"
Vitamin D_3	2	m.i.u/ton

* As menadione sodium bisulphite complex.

** This addition is of doubtful significance.
Biotin is not considered to be an essential addition.

Please let us know if you have any queries and if we can help further.

Should you favour us with any initial instructions we should like two days for manufacture and delivery.

Yours sincerely,
p.p. E.B. BRADSHAW & SONS LIMITED.

Director

APPENDIX II

Correspondence from Dr. M. Leon, and Professor H. Moltz.

McMASTER UNIVERSITY
Department of Psychology
1280 Main Street West, Hamilton, Ontario, L8S 4K1
Telephone: 525-9140 Local 4345

May 28th, 1976.

Mrs. F. M. Clarke,
Department of Psychology,
The University,
Hull, N. Humberside
England HU6 7RX

Dear Mrs. Clarke:

Thank you for your interest in the maternal pheromone. I will try to answer your questions as best as I can and I hope these answers will facilitate your research program:

1) The airflow is 55 l/min in our apparatus, perhaps the aerodynamics of our boxes are different. I would suggest blowing cigar smoke into a goal chamber and watching its rate of clearance toward the start box. In fact, no airflow is needed to find the preference.

2) Rats are tested in the afternoon, between 1:00 - 4:00 p.m. They are on a 12:12 LD schedule with no reversal of the dark phase.

3) Unfortunately, neither learning experiments nor perception experiments deal with the concept of "no choice", hence no convenient statistical tests have been formulated with that in mind. The best one can do is to perform a chi-square test comparing the choice of individual pups from separate litters to an equal number of pups presented with two empty goal boxes. Since this procedure would increase the N by a factor of 12 in my case, I have chosen to split the "no choices" between positive responses to perform the chi-square on that comparison using 50% as the expected proportion of responses. The effects are strong enough to stand the weak statistics. Most recently, I have begun to deal realistically with litter covariance and utilized the procedures outlined by Abbey and Howard (1972) and have compared experimental groups to control groups, rather than evaluate preferences within groups.

. . . . /2

4) Hooded rats produce less anal excreta in a 3 hour period than Wistars (see Galef and Heiber, JCPP in press) and therefore, the relative attraction for the mother compared to virgins is somewhat less pronounced than in the Wistars that I use. The hooded pups are also less delibrate in their choicethan Wistars. They tend to run wildly in the test situation and thus increase the noise in the data. Their response can be altered by daily handling.

5) Humans have no trouble detecting the caecotrophe odor as it is a pungent, unique odor. Mothers do smell differently from virgins. I might add that we use Purina Lab Chow which, given the odor's diet dependency, is highly effective in allowing odor production. Some lab diets contain antibiotics and may thereby inhibit microbial synthesis of the pheromone.

We are currently working on the ontogeny and dissolution of the pheromonal bond in the pups and will be putting togehter those data for publication in the near future.

Please give my regards to Jim Smart if you do see him again and please dontact me if things do not imporve with your pups.

Yours truly,

Michael Leon

ML:sc

McMASTER UNIVERSITY
Department of Psychology
1280 Main Street West, Hamilton, Ontario, L8S 4K1
Telephone: 525-9140 Local 4345

August 24th, 1976.

Mrs. Clark,
Department of Psychology,
The University,
Hull HV6 7RX
England.

Dear Mrs. Clark:

Thank you for keeping me informed of your progress. Galef and Heiber (JCPP, in press) seem to find that the hooded pups are highly reactive to anal excreta--I don't understand what is different. It might be a good idea to put the whole mother in the goal box or to present fresh material directly from the cecum to see whether the mothers are synthesizing or emitting an odor. At this point, we are working on similar problems with respect to determining why the pups go to the odor. You would be more than welcome to come to Hamilton and participate in the work that's going on here. Please let me know how things are working out with your Wistars and let me know if there is anything I can do to facilitate your research programme.

Yours truly,

Michael Leon

M. Leon

ML:sc

McMASTER UNIVERSITY
Department of Psychology
1280 Main Street West, Hamilton, Ontario, L8S 4K1
Telephone: 525-9140 Local 4345

October 4th, 1976.

Mrs. Frances Clarke,
Department of Psychology,
The University,
Hull, England.
HU6 7RX

Dear Mrs. Clark:

I just returned from my vacation to find your letter and telegram and on further consideration I realize that three weeks is not enough time for you to complete a project here. You might see what we do in a typical day or two and have some discussion with my students and I, but I would think that your visit would be much more profitable if you could arrange a long enough stay to be able to complete an experiment here.

At that time of year student accomodations are not readily available.and I will be teacing three courses which might make a visit expensive and even unprofitable for you. Perhaps a visit might be put off until it could be made more worthwhile to both of us.

Yours truly,

Michael Leon

ML:sc

McMASTER UNIVERSITY
Department of Psychology
1280 Main Street West, Hamilton, Ontario, L8S 4K1
Telephone: 525-9140 Local 4345

December 3, 1976

Mrs. F.M. Clarke,
Department of Psychology,
The University,
Hull, England
HU6 7RX

Dear Mrs. Hull,

I have been ill for the past two months and have not been able to keep up with my correspondence. In addition, I have lost more time this year than I can afford to loose, and my commitments for the coming months must be toward my own graduate students and my other responsibilities that I have been unable to fulfill in the past months. I therefore do not want to assume any more than I can handle and I am afraid that your visit would be taxing. Perhaps it would be possible for you to visit another laboratory this year.

With respect to your experimental problems might I suggest that you use the simplest situation - that of soiled vs. unsoiled bedding in a box-and if your pups do not respond, I really could not say what to do. I hope your work will soon be successful.

Yours truly,

Michael Leon
Assistant Professor

ML/rb

THE UNIVERSITY OF CHICAGO

DEPARTMENT OF BEHAVIORAL SCIENCES

COMMITTEE ON BIOPSYCHOLOGY
GREEN HALL • 5848 SOUTH UNIVERSITY AVENUE
CHICAGO • ILLINOIS 60637

3 January 1977

Ms. F.M. Clarke
Department of Psychology
The University
Hull, England HU6 7RX

Dear Ms. Clarke:

I should be delighted to have you visit my laboratory in April to discuss the maternal pheromone. And be assured that it would be no trouble at all for me to tell you what we are currently doing and what we have planned for the future. In other words, you needn't "merge in with the general graduate scene"--you would be a welcome visitor, and you may stay as long as you wish.

If I can be of any help in arranging accommodations for you, please do not hesitate to ask.

Yours sincerely,

Howard Moltz
Professor

HM:lt

APPENDIX III

SMALL ANIMAL IMMOBILON

Small Animal Immobilon contains a mixture of etorphine hydrochloride and the tranquilliser methotrimeprazine. The narcosis with analgesia which it brings about can be rapidly reversed by the use of the specific antagonist, Small Animal Revivon, which contains diprenorphine hydrochloride, a specific etorphine antagonist.

The manufacturers, Messrs. Reckitt and Colman, Hull, do not supply instructions for the use of the drug with rats, but it was found that a drug dosage of 1 ml per kilogram body weight induced anaesthesia in lactating females within about 2 minutes of intramuscular injection. The effect of this dosage level lasted for 1½ - 2 hours unless the antidote was given.

References

Small Animal Immobilon, Small Animal Revivon; Instructions for use. Messrs. Reckitt and Coleman Phamaceutical Division, Hull.

Blane, G.F., Boura, A.L.A., Fitzgerald, A.E., and Lister,R.E. (1967). Actions of etorphine hydrochloride (M.99). A potent morphine-like agent. Br. J. pharm. Chemoth., 30, 11 - 22.