Ann. Zool. Fennici 41: 765–787 ISSN 0003-455X
Helsinki 20 December 2004

Individual recognition by scent

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Received 4 Oct. 2004, revised version received 4 Nov. 2004, accepted 22 Oct. 2004

Thom, M. D. & Hurst, J. L. 2004: Individual recognition by scent. — *Ann. Zool. Fennici* 41: 765–787.

Individual recognition requires the ability to discriminate multiple animals according to their unique features. How animals do this will depend on the relative benefits to the evaluator and the cue-bearer; individuals may be discriminated either by the use of non-specific cues that are sufficiently variable to allow individual identification, or by a specific, evolved signal. Individuality information may be coded in a number of ways, although scent appears to be the primary modality for individual recognition in many species. We discuss the cue characteristics necessary to code individuality reliably, and review the bioassays that have been used to assess individual recognition ability. Although there is much indirect evidence for individual recognition by scent, many of the widely-used paradigms focus on the detectability of differences between odours rather than their function, or are compromised by the differing familiarity of odours. Further work should concentrate instead on functional assays that can disentangle odour discrimination from the role of odours in communicating individuality.

Individual recognition and scent cues

Individual recognition

Most animal species can discriminate between their conspecifics on the basis of a number of characteristics, which may include sex, reproductive status, dominance rank, familiarity, group membership, kinship and individual identity. With the exception of individual identity, each of these characteristics represents a class that may contain more than one animal. Conspecifics can usually be assigned to an appropriate class using a relatively simple distinction between a few — often only two — alternatives, such as "familiar" *vs*. "unfamiliar", "male" *vs*. "female", "group member" *vs*. "non-group member". By comparison, individual recognition requires a number of animals to be identified uniquely. Because this calls for the assessment of many unique cues, it is likely to be a much more complex task requiring specific perception and discrimination abilities (*see* Mateo 2004). Nevertheless, acquiring the capacity to discriminate individual conspecifics might bring considerable benefits, particularly if animals can remember and use information from previous encounters to moderate future responses towards the same individual. For example, recognizing individual identity could allow animals to avoid encounters with individuals to which they have previously lost contests, or to recognize long-term mating partners. Although the capacity to identify individuals should develop in such situations, relatively little is known about how widespread

true individual recognition is. The first step in assessing individual recognition is to identify the means by which this information is transmitted.

Incidental cues versus evolved signals

For individual recognition to take place, each animal to be identified must carry its own unique identity information (the expression component of recognition, *see* Tsutsui 2004). How this information is coded will depend in part on whether individuals benefit from advertising their identity. If there is no advantage to being recognized, animals are unlikely to deliberately advertise their identity and those attempting to discriminate (the evaluators) must do so without the benefit of cooperation from the cue-bearers. In this case, general characteristics of the individual may be seconded to a role in individual recognition. Humans, for example, are able to discriminate individuals using broad characteristics, such as gait or clothing, when the primary individual identity signal — the face — is obscured (Bruce & Young 1986). These general characteristics may primarily serve other signalling functions or they may have no role in signalling, but can nevertheless be utilised by other conspecifics and may be used to discriminate between individuals. This type of interaction is sometimes described as eavesdropping (Bradbury & Vehrencamp 1998), since the evaluator is detecting and using information that is not actively signalled to it by the cue-bearer.

While individual recognition may be possible without any cooperation from the animal being identified, there are many situations in which animals could benefit from actively advertising their individual identity to conspecifics. Although predicting when the benefits of signalling identity outweigh the costs can be difficult (Johnstone 1997), such advertisement is likely to be particularly advantageous in species with complex social interactions, where the ability to identify individuals allows the recognition of long-term partners, the establishment of dominance hierarchies or other competitive relationships based on individual identity, or the maintenance of delayed reciprocal altruism. Where individual recognition benefits both participants, selection should ensure that identity is reliably characterised and easy to assess. This could be achieved through the evolution of an individual identity signal if existing incidental cues are not sufficiently reliable for unambiguous identification, or do not allow efficient recognition in contexts where recognition is important (for example, at a distance or in the dark).

Information required for individual recognition

Any individuality cue, whether it is based on incidental information or an evolved signal, should exhibit certain key characteristics in order to fulfil the requirement for reliability. Firstly, it should be relatively independent from other background variation: that is, it should be sufficiently distinctive to be easily and reliably disentangled from all other types of information (Halpin 1986). For example, humans discriminate between familiar individuals on the basis of their facial characteristics, an ability that appears to be relatively robust to changes in facial expression (Posamentier & Abdi 2003). Facial expressions are a source of short-term information about a person's current emotional state, but the ability to recognize the individual producing different facial expressions is largely independent of such changes.

Secondly, reliable individuality signals should exhibit a high degree of diversity between individuals. However, it is usually unnecessary for every individual of a species to have a unique cue, because geographic and temporal isolation ensure that the majority of individuals of a species will never meet. Thus, the diversity required will depend on each species' social system and contact networks. Some degree of error may also be acceptable — for instance we know from personal experience that there are occasional errors in individual recognition among humans, but the system is sufficiently accurate to maintain a large range of social functions based on individuality. The error rate depends on the combinatorial diversity of the cues available, with precision being maximised when each individual has a unique cue (Dale *et al*. 2001).

Finally, identity cues should exhibit temporal stability. While age, reproductive status, health

and social status vary through time, identity (species, sex, individual) remains constant. In order to maintain the association between the individual animal and its identity signal, the signal must remain stable despite variation in other characteristics reflecting the animal's current status and environment (it should be condition-independent: Dale *et al*. 2001). It is not essential for an individual identity cue to remain fixed throughout life, as long as any changes take place sufficiently gradually to allow the evaluators to adjust their recognition template (*see* Liebert & Starks 2004). The degree of stability required is thus a function of the rate of change in relation to opportunities to update the signal — a signal might be able to change more rapidly where frequent encounters ensure that each evaluator's template remains updated, although a signal fixed for life would never need to be updated.

Any cue with these characteristics could be considered reliable for the purposes of individual recognition, whether this cue arises incidentally or has evolved specifically to provide a signal of identity. Signals that provide reliable information about the individual identity of the cue-bearer are likely to be found in a wide range of situations, but they are likely to be particularly important in cases where the signal is physically separated from the cue-bearer, as is often the case in communication through scent.

The use of scent for signalling individual identity

Animals release odours from many sources including a wide range of scent glands and in faecal and urinary excretions (e.g. Brown 1979, 1995, Brown & Macdonald 1985). In many cases, scents are deliberately placed in the environment in the form of scent marks to provide information over a prolonged period of time, even when the owner is elsewhere. To provide information about the specific scent owner, a scent mark needs to include reliable and long-lasting information about the owner's identity when no other cues are available. For example, scent marks are used by many species to signal their ownership of territories, both to defend them from potential competitors and to attract potential mates. Only animals that can successfully protect their territory are able to ensure that their scent marks predominate (Gosling 1982) and, in order to ensure that their marks remain predominant, territory owners quickly countermark any scent marks left by intruders (Johnson 1973). The original mark, the intruder mark, and the countermark all persist in the environment, providing a lasting record of both the challenge and the response by the territory owner as long as the different owners of the scent marks can be identified (Hurst & Beynon 2004). Because scent marks are deliberately deposited as signals from the scent owner, we might expect to see the evolution of signal components that allow both efficient and reliable communication of the cue-bearer's identity (species, sex and individual).

Scent marks carry both fixed (genomic) and variable (metabolic) information about the owner. Metabolic fluctuations resulting, for example, from changes in health, reproductive status, diet, and social rank may all result in changes to the odour profile of an individual's scent marks. In comparison, the invariant information contained in scent relates to genetically fixed characteristics such as species, sex, and individual identity. For example, information on species and sex in the mouse are inherently coded in male mouse-specific signalling volatiles expressed under androgen control that can easily be distinguished against the complex set of metabolites excreted in mouse urine (Harvey *et al*. 1989). However, it is not yet established for any species whether there are also specific genetically-coded individuality signals in scent that are encoded independently of other variation. Detecting an individual identity signal is complicated by the fact that animals may also be able to discriminate differences between individuals using incidental cues, which might include both metabolic and genetically determined scents. For example, the combination of sex, familiarity, age, and dominance status could be sufficient to assign approximate identities to several individuals in a small population. However the fluctuating nature of metabolic cues is likely to make a system based entirely on such cues relatively unstable, and we might expect relatively high rates of identification error as a result. Further-

more, variable metabolic cues also carry other useful information which is of interest in its own right. Animals need to recognise scent owner identity (species, sex and individual) and associate this with additional information about the owner's current status. A genetically-determined cue offers distinct advantages, overcoming both the problems of instability and of dual signalling roles. However, while many genes are likely to contribute indirectly to differences in the scents expressed by individuals through influencing levels of metabolites, these same components are also likely to be influenced by current physiological state and may not be a stable characteristic of the animal despite a genetic influence (Humphries *et al*. 1999, Hurst & Beynon 2004). To provide the necessary stability, a reliable scent cue ideally needs to be both genetically-determined and expressed independently of metabolic and environmental fluctuations in order to avoid the need to continually update the recognition template.

Assessing individual recognition

Although there is a strong basis for predicting that many species may be able to discriminate between individuals rather than classes of conspecifics, it has proven difficult to determine empirically whether animals are able to identify individuals and which cues they use to do so. This is largely because of the difficulty in discriminating between responses to general changes in odour, and responses that are specifically related to individual recognition. While the need to distinguish between the two may seem self evident, it is not easy to eliminate potentially confounding variables. A particularly common problem is differing familiarity of the test scents to the subject. Under these circumstances, a difference in response to one scent compared with another could indicate recognition of familiarity rather than individuality. Designing experimental paradigms that eliminate differences in familiarity has proven particularly difficult. In fact, it is virtually impossible to eliminate all differences between a pair of scents apart from those due to an individuality signal. This difficulty is likely to be particularly problematic in species where incidental cues are involved in the assessment of individual identity. Because incidental cues are likely to code a range of variable information, detection of a change in these cues between two individuals does not necessarily indicate individual recognition. Animals are likely to be sensitive to changes in a range of scents or scent components, many of which will impart information of considerable importance about the scent owner but are not concerned with the owner's individual identity. Experimental paradigms thus need to distinguish between a response that is elicited merely by a change in scent, and a response which specifically indicates recognition of individual identity. In this context, functional responses that involve a reactive behavioural or physiological response which specifically relates to individual recognition are more informative than responses that reflect simple discrimination between scents. Nevertheless, responses that are based only on scent discrimination or investigative behaviour have been used widely in the assessment of individual recognition, and we begin by discussing these types of test before considering functional paradigms.

Non-functional paradigms

Operant conditioning

By repeatedly presenting two odour stimuli simultaneously, one of which is coupled with a reward, it is possible to train rodents to respond differentially to a pair of odours. Training often begins with simple odours, such as juniper and cinnamon, or mouse odours from pairs of strains that are genetically very different. Subsequently, testing moves on to pairs of strains with more subtle genetic differences at the loci of interest. The training regime generally consists of depriving animals of water for several hours, then allowing access to a water droplet as a reward for a correct response. Often many hundreds of trials are required to achieve the predetermined concordance level, which is usually set at around 80% or 90% (e.g. Yamaguchi *et al*. 1981, Yamazaki *et al*. 1990, 1994, Bard *et al*. 2000).

Operant conditioning experiments have provided some of the best evidence for the ability

of rodents to discriminate odours. These studies have particular value in determining the detection thresholds of various stimuli, and in this context they have been invaluable in demonstrating the acute sensitivity of mice and rats to small changes in both metabolically and genetically associated odours. In fact, these tests have demonstrated olfactory acuity to be of such sensitivity that the vast majority of odours tested have been successfully discriminated (*see* Appendix 1 for some of the major findings resulting from using this technique).

Habituation–dishabituation and habituation–discrimination

The habituation–dishabituation bioassay provides an attractive alternative to operant conditioning, and is perceived as being more natural than operant conditioning since it relies on spontaneous rather than trained behavioural responses (Brown *et al*. 1990, Penn & Potts 1998b, Carroll *et al*. 2002). In this procedure, a stimulus is presented repeatedly until a habituation response is observed — that is, the investigation of the stimulus declines as it increases in familiarity. A test stimulus is then presented. If the subject detects a difference in the new scent as compared with the habituation scent, investigation increases to levels close to — but generally lower than — those observed in the first habituation presentation. This is the dishabituation response. The control test generally consists of a test sample derived from animals genetically identical to those that provided the habituation scent and maintained under similar conditions. To determine whether the response to the test odour is greater than that to the control, the magnitude of the dishabituation response is compared between the two tests. This is necessary since the technique is extremely sensitive to small odour changes, and subjects typically dishabituate even to control samples (e.g. Penn & Potts 1998b). To reduce this response, each sample usually consists of pooled urine collected from several genetically identical animals. This has the effect of reducing the dishabituation to the control test, although even after pooling some residual level of response usually remains (Penn & Potts 1998b). In a variation on this procedure, the final test sample and the control sample are presented simultaneously. In this design, called habituation–discrimination, the responses to the habituated test scent and the control scent can be compared directly within a single trial (e.g. Johnston & Jernigan 1994). Both the habituation–discrimination and the more widely-used habituation–dishabituation tests have been used to demonstrate that both mice and rats will respond spontaneously to a wide variety of subtle genetic and metabolic changes in odour (Appendix 2).

Limitations of non-functional tests

Operant conditioning studies conclude that two odours can be discriminated if subjects visit a rewarded odour at greater than chance levels. Although a powerful method for assessing the detectability of odour differences, it does rely on at least two markedly unnatural conditions. Firstly, the subjects are trained to associate a reward with a particular odour, a process which often takes several hundred trials to achieve. Apart from the artificial nature of this process, there is the additional problem that with sufficient training mammals — including humans — can learn to detect at least some types of odours to which they are not normally sensitive (Wang *et al*. 1993). Secondly, subjects are typically water-deprived, and thus unusually highly motivated to acquire a water reward for compliance. How this compares with the motivation to detect small odour differences under natural circumstances is unknown, but it may be significant that even under the powerful motivating influence of acute thirst, mice and rats generally find the operant conditioning task difficult (as evidenced by the large number of training trials required). The circumstances of the operant conditioning paradigm are far removed from any natural functional context — a limitation that has repeatedly been pointed out (e.g. Halpin 1980, 1986, Schellinck & Brown 1992, Penn & Potts 1998b). Furthermore, the non-functional nature of the test means that operant conditioning studies measure only whether an odour difference is *detectable* after intensive training, and not how relevant this difference is for individual recogni-

tion. Operant conditioning studies nevertheless remain effective in determining whether pairs of odours can be detected (e.g. Lai & Johnston 2002, Osada *et al*. 2003), and may act as a first step towards assessing any role such odours may play in social communication.

Habituation–dishabituation and habituation–discrimination methods remove the complicating factors of training and motivation enhanced by water deprivation, but similarly suffer from a lack of functional context. In fact, by relying entirely on variation in a spontaneous investigation response, the habituation–dishabituation paradigm is based on somewhat less robust assumptions. In the majority of cases subjects dishabituate to the control as well as the test samples, illustrating the extreme sensitivity of the test — even genetically identical individuals from the same strain are often discriminated (e.g. Brown *et al*. 1990, Penn & Potts 1998b), and when urine from multiple individuals of the same strain is pooled to control for non-genetic variation, some degree of dishabituation still occurs (Penn & Potts 1998b, Carroll *et al*. 2002). The important point here is that rodents respond to *virtually every odour tested* using this paradigm, regardless of whether these odours are involved in individual recognition. So can this method be used to assess which odours are important for individual recognition? The usual mechanism for assessing the relative importance of odours is to compare the magnitude of the dishabituation response in test and control trials. Dishabituation to the control odours from genetically-identical individuals represents a response to non-genetic variable background information, which, while it may carry useful information on an animal's current status, is unlikely to be the basis of a stable individuality signal. An increased magnitude of response to strains that are genetically distinct is often interpreted as evidence for individual recognition, signalled by whichever genetic component differed between the pair of scents under test. However, it is inevitable that adding genetically-induced odour differences to those resulting from non-genetic sources will increase the magnitude of the disparity between odours, and hence the strength of the investigation response. If several genetic differences each lead to increased response over the control, the habituation–dishabituation test offers no mechanism for determining their relative importance for detecting particular information, except by ranking the size of the dishabituation response. Unfortunately this assumes an unsupported relationship between dishabituation and relevance to individual recognition.

The problem of assessing the relative importance of two dishabituation responses highlights a broader limitation of the habituation–dishabituation paradigm. This type of test is based entirely on the measurement of investigation, which is an inappropriate measure for assessing recognition. Investigation is associated with information gathering, which is part of the perception component of scent assessment (Mateo 2004). Increased investigation thus implies the recognition of *novelty* in a stimulus which stimulates increased information gathering to allow perception of the cues, but unfortunately it does not tell us anything about the nature of the new information, nor its interpretation by the subject. A wide range of metabolically- and genetically-influenced scents have elicited a response in habituation–dishabituation tests (Appendix 2). In order to determine which components of scent have meaning in individual recognition, we need to assess the action taken by the animal after it has perceived information on the identity of the scent owner (the action component, *see* Liebert & Starks 2004). Only by looking at specific functional responses elicited by individual recognition can we discriminate between scents that are novel but irrelevant, scents that provide information about the status of the scent owner, and scents that allow recognition of individual scent owners. Paradigms that attempt to assess the functional significance of odours by assessing behavioural or physiological responses have been developed, and these are discussed below.

Mate choice and pregnancy block

Some of the first evidence that rodents discriminate among their conspecifics came from mate choice and kin selection studies. For example, mice of both sexes generally prefer mating partners from different inbred strains to their own (Yamazaki *et al*. 1976, 1978, Egid & Brown

1989), while females nest preferentially with females genetically identical to themselves over those from a different strain (Manning *et al.* 1992). Clearly both of these abilities could be explained without invoking individual recognition, as animals could select both mating and nesting partners on the basis of simple binary categorization into 'different from self (or kin)' and 'similar to self (or kin)'. Indeed, mate choice studies do not generally test for the ability to recognise individuals, but rather the ability to classify conspecifics according to the sharing of particular characteristics (*see* Lewis *et al.* 2004). Exceptions to this lie in those cases where potential partners do not differ from each other on the basis of their relatedness or familiarity to the subject, but have other characteristics which allow them to be uniquely identified. For example, brief exposure to a male odour increases female preference for that male in a subsequent binary test, an effect that is strong enough to override the usual bias against parasitized males (Kavaliers *et al.* 2003). Although this particular design still suffers from the problem that males differ in their familiarity to the female (and indeed was not designed to test individual recognition), further work could refine such mate choice tests to overcome these difficulties.

Females tend to prefer males that advertise their territory ownership by maintaining exclusive scent-mark coverage (Rich & Hurst 1998, 1999; also *see* below). This requires females to exercise a choice of individual males, a behaviour which presents the opportunity to develop alternative mate-choice based bioassays of individual recognition. For example, following exposure to a pair of scent-marked male territories, females express a subsequent preference for males whose territory is either exclusively marked by the owner, or where any intruder marks have been counter-marked, over those males owning territories that have been counter-marked by an intruder (Rich & Hurst 1998, 1999). Similarly, in species where males physically overmark intruder scent marks (placing their own scent directly on top of the intruder scent, rather than adjacent to it), females can discriminate between the original mark and the overmark, and again they prefer the overmarking male (Johnston *et al.* 1997). This bioassay overcomes the problem of unequal familiarity of subjects, since both the intruder mark and the countermark are of equal familiarity to the female. The results imply that females have recognized the individual identities of the two territory owners in order to discriminate, and that this recognition is independent of any differences in the familiarity of the male scents.

Pregnancy block, or the Bruce effect, has been used quite extensively to assess the scent cues that females use to recognize a familiar partner with which they have recently mated and the neurophysiological mechanism underlying individual recognition. If a pregnant female is separated from the stud male with which she has mated during the first few days after copulation and exposed to the odours of a novel male, a significant proportion of pregnancies fail (Bruce 1959; reviewed by Brennan & Peele 2003). Shortly after mating, females form an olfactory memory of the stud male in the accessory olfactory bulb (Brennan *et al.* 1990). Subsequent exposure to scent from an unfamiliar male of a different strain to the stud male within 5 days of mating disrupts prolactin release from the anterior pituitary, causing failure of embryo implantation in the uterus. By contrast, pregnancy is not blocked by the scent of males that are genetically identical to the stud male. This test therefore has been used to assess the genetically-determined olfactory cue by which a female recognises the stud male (Yamazaki *et al.* 1983b, 1989, Peele *et al.* 2003, Leinders-Zufall *et al.* 2004). The pregnancy block paradigm has an advantage over non-functional tests, since it measures a natural physiological response on encountering scents from genetically-distinct individuals. As a result, it can be applied to address the ability of females to discriminate familiar individuals (with which they have recently mated) from unfamiliar males, the molecular basis of the scents used in this recognition, and the neurophysiological mechanisms underlying the recognition process.

However, some caution has been voiced concerning the functional significance of the pregnancy block response, which is not as clear-cut as is sometimes suggested. Firstly, there is no useful model of how pregnancy block might operate in the wild. Although it has been suggested that the mechanism allows females to upgrade their choice of mate should a new male

take over the local territory, this does not explain the observations that subordinate males are just as effective at blocking pregnancy as dominant males and exposure to subordinate males can even block pregnancies sired by dominant males (Labov 1981) while scent from males genetically identical to the female will block pregnancies sired by unrelated males (Parkes & Bruce 1961). In comparison, in pre-copulatory tests females show a strong and predictable preference for dominant over subordinate males, and for unrelated over closely related males. Additionally, the few field tests conducted so far have failed to demonstrate the existence of pregnancy block in semi-natural rodent populations (de la Maza *et al*. 1999, Mahady & Wolff 2002, Wolff 2003). Secondly, the pregnancy block response is not specific to exposure to odours from different individual males, and may also be a response to novelty or stress rather than individuality. For example, a variety of physical stimuli have been shown to cause pregnancy block (Weir & de Fries 1963). The response is strongest among sexually naïve females that had not previously encountered adult males, so that the novelty of the second male odour encountered might possibly be responsible for the effect rather than individual recognition per se. Finally, although pregnancy block measures a natural physiological response, the test itself is not particularly natural. The test usually involves an exaggerated stimulation of the olfactory system through the application of large quantities of urine to the nose of the mated female, a situation that seems unlikely to occur in nature. Nevertheless, pregnancy block provides a very useful test to address the neural mechanism in the vomeronasal system that underlies recognition of a familiar stud male compared to a novel male.

A physiological response known as the Coolidge effect describes the phenomenon whereby males mated to satiety with one female will display renewed sexual interest when presented with a novel female (Dewsbury 1981). This mechanism has been interpreted as evidence for individual recognition (e.g. Petrulis & Eichenbaum 2003). A test of individual recognition based on the Coolidge effect would benefit from the spontaneity of this behaviour, which has distinct functional benefits to the male. However any such bioassay suffers from the recurring problem of unequal familiarity — novel and previously-mated females are not of equivalent familiarity to the subject. While it might be possible to alleviate this to some extent by allowing prior contact with both females, females still fall into two distinct classes of 'previously mated' and 'not previously mated'. There also remains a possibility that the act of mating may in some way influence the female's odour (perhaps by the deposition of the male's own scent on to the female).

Territorial countermarking

Scent marks in the environment continue to provide information to other conspecifics when the owner is absent, and are used by territory owners to demonstrate their competitive superiority by ensuring that their own marks are always the freshest and most abundant in their territory. This is a reliable signal of competitive ability, because success in defending the territory is a prerequisite for maintaining scent marking coverage of the defended area. This signal of competitive ability makes successful males more attractive to females (Engel 1990, Rich & Hurst 1998, 1999). As a result, territorial animals are highly motivated to defend their ownership of territories through scent marking, and territory holders immediately countermark the marks of intruding males in order to emphasise ownership (Johnson 1973, Hurst & Rich 1999). However for territory marks to demonstrate ownership and competitive ability effectively, they must contain an individuality signal that allows potential competitors and females to recognise the territory owner. Without this identity information, all the benefits of scent marking are lost. For this reason, territorial scent marks are an ideal cue in which to look for individual identity signals.

Furthermore, this counter-marking system represents a functional assay of individual ownership recognition (Hurst *et al*. 2001). While mice countermark the scent marks of other males, they investigate but do not countermark in response to their own scent. The fact that mice respond to intruder but not to own marks indicates that they can discriminate ownership at least into the binary categories of 'self' and

'not self'. However the countermarking response can also be used to assess more sophisticated recognition tasks, including that of individual recognition. Because each territory typically borders those of several other males (Crowcroft & Rowe 1963, Wolff 1985, Hurst 1987), mice may benefit from an ability to identify each individual neighbour by scent since this would allow them to determine ownership of competitors' marks deposited in their territory. In laboratory enclosures, territorial male mice can correctly identify an intruder's scent mark and will countermark next to the neighbour from which the scent mark originated (J. L. Hurst & R. Frost unpubl. data).

The chemical basis of individual identity

The tests described above have been used to address the odour sources and the mechanisms underlying individual recognition in a number of rodent species (e.g. golden hamsters *Mesocricetus auratus*: Johnston & Bullock 2001, Petrulis & Eichenbaum 2003; mound-building mice *Mus spicilegus*: Gouat *et al*. 1998; giant kangaroo rats *Dipodomys ingens*: Murdock & Randall 2001; prairie voles *Microtus ochrogaster*: Mahady & Wolff 2002; gray-tailed voles *Microtus canicaudus*: de la Maza *et al*. 1999). However, by far the bulk of research has focused on the house mouse (*Mus domesticus*) and brown rat (*Rattus norvegicus*), partly due to the ready availability of genetically-defined inbred strains that differ from each other at specific loci. Here we review evidence for the chemical basis of cues used in individual recognition, largely derived from studies of these two main model species.

MHC-associated odours

Urine is the primary source of scent cues in the mouse and rat, and animals of both sexes use urine marks for conspecific communication (Dagg *et al*. 1971, Brown 1975, 1977, Maruniak *et al*. 1975). Many genetic loci influence the complex set of volatiles expressed in rodent urine (Boyse *et al*. 1987, Beauchamp *et al*. 1990, Eggert *et al*. 1996), which is made up of metabolic by-products and chemical components specifically manufactured and added to urine to act as chemical signals. An individual-specific cue needs to be sufficiently diverse to ensure that animals within the same local group, many of which are likely to be related and from a limited genetic pool, have different and easily discriminated scent cues. Much attention has therefore focused on the highly polymorphic major histocompatibility complex (MHC, called H-2 in the mouse and RT1 in the rat) as the main source of individual-specific genetically-determined scents ever since Yamazaki and colleagues showed that mice can discriminate urinary odours from MHC congenic strains that differ only at loci within the MHC region (Yamazaki *et al*. 1982, 1983a). Rats have a similar ability to discriminate between urine scents of MHC congenic strains of both mice (Brown *et al*. 1996) and other rats (Brown *et al*. 1989, 1990, Schellinck *et al*. 1991). Indeed, subsequent studies have revealed that MHC type influences odours in a wide range of vertebrates (e.g. fish: Aeschlimann *et al*. 2003, Olsen *et al*. 2002; lizards: Olsson *et al*. 2003; birds: Zelano & Edwards 2002; humans: Porter & Moore 1981, Wedekind *et al*. 1995, Jacob *et al*. 2002).

Given the extraordinary degree of polymorphism of the MHC, and the influence of MHC on odours across a broad range of species, it has been suggested that MHC-associated odours might be used both for the assessment of close genetic relatedness (through shared MHC odours) and for individual recognition (through differences in MHC odours expressed by animals that are not closely related) (Yamaguchi *et al*. 1981, Brown *et al*. 1987, Beauchamp *et al*. 1990, Penn & Potts 1998b). Studies using functional tests of mate choice or kin recognition have confirmed that MHC-associated odours appear to play a role in the assessment of close genetic relatedness (reviewed by Penn & Potts 1999, Penn 2002). For example, female mice prefer to nest communally with other closely related females, favouring females of the same MHC type as themselves (Manning *et al*. 1992). Conversely in mate choice studies, mice prefer the scents of potential mates of different MHC type to those of their parents, experienced during rearing (Yamazaki *et al*. 1978, Egid & Brown 1989, Penn & Potts 1998a). This leads to MHC

disassortative mate preferences (Potts *et al*. 1991), a mechanism that might also operate among humans (Wedekind *et al*. 1995, Jacobs *et al*. 2002). However, it is not clear whether animals use MHC type as a marker of general relatedness or, more specifically, to promote diversity at the MHC (reviewed by Penn & Potts 1999). In strong contrast to individual recognition, these tests all demonstrate that animals are able to recognise familiarity in scents *across* different individuals, assigning those individuals that match their own scent, or the highly familiar scent on which they imprinted during rearing, to one class while all other individuals are assigned to a different class.

Because unrelated individuals are all likely to express different MHC types, it has widely been assumed that MHC also provides the main source of variation in odours that are used for individual recognition. However, the significance of MHC in individual recognition remains unclear. This is due both to uncertainty about the mechanism of signalling identity through MHC associated odours, and a shortage of evidence demonstrating a link between MHC odours and individual recognition in a functionally meaningful context. Although there is considerable evidence for the ability to discriminate odours according to MHC type from both operant conditioning (Appendix 1) and habituation–dishabituation (Appendix 2) studies, there is very limited evidence for the functional use of such information in individual recognition. One functional test of individual recognition that has been applied is the ability of MHC-associated odours to induce pregnancy block. Scent from an unfamiliar strain that differs from the stud male only at MHC is sufficient to induce failure of pregnancy (Yamazaki *et al*. 1983b, 1986). However, the response differs in two critical ways from that induced by different strain males in many other studies, raising doubts about the mechanism of response to MHC-associated scents. While many studies have shown that pregnancy block is a specific response to contact with androgen-dependent scents from unfamiliar males (e.g. Bruce 1960, Dominic 1965, Hoppe 1975), scent from an unfamiliar MHC congenic strain induces pregnancy block whether scents are from males or from females (Yamazaki *et al*. 1983b). Further, recognition of an unfamiliar male's scent is mediated through the vomeronasal system and requires contact with the male or with male urine odours (Brennan & Peele 2003); airborne volatiles alone are not normally sufficient to induce pregnancy block (Dominic 1966, Rajendren & Dominic 1984). However, airborne urinary odours from a strain MHC congenic to the stud male appear to be just as effective as direct contact (Yamazaki *et al*. 1983b). This unusual response to airborne odours from mice of different MHC type to the stud male might thus be more consistent with a general stress response on encountering an unfamiliar mouse odour through the main olfactory system rather than recognition of scent from a different individual to the stud male through the vomeronasal system.

An individual identity cue needs to be not only highly polymorphic, but should also be a stable characteristic of the individual that can be discriminated independently of changes in individual status and environment. While MHC fulfils the requirement of a polymorphic cue that will differ at least between unrelated individuals (close relatives may share the same MHC-associated odours as demonstrated by mate choice and kin recognition studies), the stability of MHC-associated odours and independence from other non-genetic variation depends on the molecular basis of MHC odours.

The mechanism underlying the production of MHC-associated odours has not yet been elucidated, but MHC type is detected through airborne odours that appear to consist of a complex set of volatile metabolites that are bound and released by urinary proteins (Singer *et al*. 1993, 1997). Three mechanisms have been proposed to explain how these MHC-associated odours might be produced. An early hypothesis suggested that MHC type determined the commensal bacterial flora of the skin, urinary tract and gut due to the role of MHC in the immune response; thus, individual MHC types would be associated with unique flora. As a result, the volatile odorants in urine might arise as secondary metabolites derived from these bacteria (Howard 1977, Brown 1995). However, the composition of an individual's bacterial flora is not constant through time, and a number of experiments have shown that the immune regulation of commen-

sal flora is not necessary for the production of MHC-associated odours, thus disproving this hypothesis (reviewed by Singh 2001). Nevertheless it is still likely that MHC-dependent bacterial flora contribute to volatile odourants in rodent urine.

The "carrier hypothesis" (Singh *et al*. 1987, Singh 1999, 2001) instead proposes that large fragments of MHC proteins in urine act as odorant carriers, binding a specific profile of volatiles from the complex mixture of metabolites in urine or serum. The allelic differences that specify MHC are concentrated within the antigen-binding cleft that normally binds foreign peptides. As MHC class 1 proteins undergo degradation in urine, the antigen-binding cleft opens, resulting in the loss of the bound peptide. According to the carrier hypothesis, this vacant peptide binding site then acquires a set of small volatile molecules with a binding specificity dictated by the allelic variant. A unique set of volatile odorants would thus be selected from a common pool of metabolites in urine, to which commensal flora may also contribute; this volatile mixture is then slowly and steadily released from the carrier protein fragments to provide an individual odour signature. At present, there has been no direct test of the carrier hypothesis although results of an indirect test by Pearse-Pratt *et al*. (1999) are consistent with such a mechanism. However, a limitation of the hypothesis is that it is unclear how low molecular weight volatiles (typically 200–300 Da) could be specifically bound to class I MHC protein fragments that normally bind much larger peptides (typically around 1000 Da) (Singer *et al*. 1997).

Alternatively, MHC type is likely to have a direct influence on the pool of volatile metabolites in rodent urine that bind to urinary proteins. MHC polymorphism is known to have a wide influence on organ development, growth and hormone levels and these developmental variations may give rise to a distinctive pattern of volatile metabolites (Boyse *et al*. 1987). While MHC proteins are designed to bind peptides, another set of proteins, present in rodent urine at very high concentrations, are designed to bind and release low molecular weight odorant molecules. These proteins, termed major urinary proteins (MUPs) in the mouse or α-2u globulin in the rat, are lipocalin proteins whose only known functions are in chemical signalling. These proteins have a central cavity that binds small apolar ligands (Beynon & Hurst 2004). This cavity is flexible and is able to accommodate a wide range of small molecules including environmentally derived chemicals (Robertson *et al*. 1998), reporter molecules (Marie *et al*. 2001) and a variety of specific pheromones (Novonty *et al*. 1999, Sharrow *et al*. 2002). One of the main functions of MUPs is to act as a vehicle to elicit the slow release of pheromones from scent marks (Hurst *et al*. 1998). This might also include the binding and release of volatile metabolites influenced by MHC type. This mechanism is particularly attractive as it would provide a close physical link between the highly polymorphic pattern of volatile metabolites expressed by individuals (influenced by MHC and many other genetic loci) with the highly polymorphic pattern of involatile urinary proteins that are also expressed by individuals (*see* below). As yet, there have been no tests to distinguish between this MUP carrier hypothesis and the alternative MHC protein carrier hypothesis.

Whichever mechanism applies to the production of MHC-associated odours, MHC type appears to be characterised by a complex set of volatile metabolites. These metabolites are also likely to be influenced by variable non-genetic factors including hormonal status, diet and bacterial flora. This raises a question about how stable such odours are likely to be among animals that inhabit variable environments and how well cues indicating the individual identity of the cue-bearer could be separated from information concerning the bearer's current status. Although highly familiar odours can be recognised in a constant laboratory environment, odour discrimination tests suggest that changes in diet, for example, mask recognition of MHC type (Schellinck *et al*. 1997). Factors such as daily diet and bacterial flora picked up by individuals from the environment are likely to vary through time within an individual among opportunistic species like mice and rats, resulting in changes to the complex pattern of metabolites expressed in an individual's urine. This may limit the long-term reliability of such cues for individual recognition, although MHC scents are likely to

contribute to the recognition of familiar individuals while these scent cues remain unchanged (*see* below).

Major urinary proteins (MUPs)

Recent studies have revealed that the pattern of MUPs expressed by individual mice is also extremely polymorphic (Robertson *et al.* 1997, Pes *et al.* 1999, Payne *et al.* 2001, Beynon *et al.* 2002). MUPs appear to have ideal characteristics for providing a stable and persistent individual identity cue (Beynon *et al.* 2001) and their extreme polymorphism may have evolved for this purpose. Each individual typically expresses 7 to 14 separate MUP bands when MUPs are separated according to charge by isoelectric focusing, and there is considerable combinatorial diversity between individuals even within geographically isolated populations where genetic heterogeneity is much reduced. The pattern of MUPs expressed in urine is a fixed, genetically determined characteristic that remains the same throughout adult life regardless of status changes or alterations in food resources. Once deposited in scent marks, the involatile MUPs can also persist without degradation over many weeks or months (Hurst & Beynon 2004). Unlike MHC and other genes that influence the profile of metabolites in urine, their only known role is in chemical signalling. Using competitive countermarking as a functional assay of individual scent ownership recognition, Hurst *et al.* (2001) showed that MUP pattern was essential for the recognition of own urine marks and those of other males, despite many other genetic differences between outbred wild-derived mice. More recent tests from our laboratory have further confirmed that MUP pattern is responsible for the more general recognition of individual ownership signals from different conspecifics that are otherwise of equivalent familiarity and status (J. L. Hurst & R. Frost unpubl. data).

Behavioural, biochemical and neurophysiological evidence indicate that individual scent ownership is signalled either by non-volatile MUP-ligand complexes or by the involatile MUPs themselves rather than by volatile ligands released from MUPs. Although there is good evidence that the affinity of different MUPs for natural or reporter ligands can vary (Marie *et al.* 2001, Sharrow *et al.* 2002), comparison of the release kinetics between mouse strains that express very different MUP patterns reveals no major differences in the rate of loss of two of the main male signalling volatiles, 2-*sec*-butyl-4,5-dihydrothiazole and 2,3-dehydro-*exo*-brevicomin (Robertson *et al.* 2001). Direct nasal contact with the scent source is essential to detect the individual scent ownership signal (Luo *et al.* 2003), both to induce pregnancy block (Brennan & Peele 2003) and to stimulate competitive countermarking of another male's scent (Nevison *et al.* 2003), indicating that individual recognition involves non-volatile scent components. While airborne volatiles are detected through the main olfactory system, non-volatile scent stimuli are detected via the vomeronasal system (Halpern & Martinez-Marcos 2003). The individual recognition cue involved in pregnancy block is due largely to low molecular weight components (applied directly to the nares) but is enhanced when these are delivered in the context of urinary proteins (Peele *et al.* 2003). However, the molecular cues underlying individual recognition in pregnancy block may differ from those involved in scent mark ownership signalling. Pregnancy block requires exposure to very fresh male scents — importantly, female reproductive strategy is not influenced by exposure to male scent marks that have aged (Parkes & Bruce 1961, Rajendren & Dominic 1984). Since the pregnancy block response occurs only over a short time period (within 5 days of mating), the cues used for individual recognition need only limited temporal stability and must involve only fresh signals to ensure that pregnancy is not influenced by old scent signals that will remain in the environment over extended periods. By contrast, the ownership signal in territorial scent marks deposited in the environment needs to be long-lasting. Intruder scents that have lost ligands through natural ageing over many days, or by chemical displacement, induce as strong a countermarking response as do fresh signals (Humphries *et al.* 1999). This suggests that any MUP ligands involved in individual recognition of scent mark owners are relatively involatile and resistant to chemical displacement, or that mice are able to recognise the MUP patterns themselves. Although

vomeronasal receptors for MUPs remain to be identified, the anterior region of the accessory olfactory bulb receiving input from the vomeronasal organ respond preferentially to MUPs (and any strongly bound ligands) while the posterior region responds to the main MUP ligands 2-*sec*-butyl-4,5-dihydrothiazole and 2,3-dehydro-*exo*-brevicomin (Brennan *et al*. 1999).

An integrative model for individual recognition scents

The complex volatile and involatile components of scents such as rodent urine have different advantages and disadvantages for individual recognition. Airborne volatiles are detected much more rapidly, and at a distance, through the main olfactory system, without the requirement for physical contact with the scent source. However, while the complex volatile profile of an individual is influenced by a wide range of genes including those of the highly polymorphic MHC, this will also be influenced by a wide range of other less stable factors, as discussed above. By their very nature, volatile components are also likely to be lost at different rates from scent marks, limiting the stability and persistence of such information. By contrast, highly polymorphic involatile components such as the MUPs in the urine of house mice appear to have evolved to provide a specific and highly stable chemical signal of individual identity that is a fixed characteristic of the individual expressed independently of other factors, and is stable and persistent when deposited in scent marks. However, involatile scents are undetectable at a distance and pumping them to the vomeronasal organ requires investigation through physical contact with the scent source. While direct contact with a scent mark (detected through the release of volatiles) is relatively easy if time consuming to achieve, physical contact with other conspecifics can be considerably more dangerous particularly if these are competitors.

Hurst and Beynon (2004) have suggested an integrative model for the recognition of individuals through scents that is based on the opportunity for animals to learn associations between volatile and involatile scent profiles. Whenever animals detect some unfamiliarity in their scent environment through airborne volatiles, they generally approach the scent source to investigate closely, providing them with the opportunity to also detect involatile components of the scent. Any difference in volatile scents compared with those they are highly familiar with is closely investigated, providing animals with the opportunity to detect involatile identity signals such as MUPs. While investigating an involatile signal detected through the vomeronasal system, animals have the opportunity to associate this with the complex volatile scent profile simultaneously detected through the main olfactory system (Guo *et al*. 1997). If they encounter the same complex volatile scent profile again, a memory of the involatile individual scent signal would obviate the need to contact the scent source to gain this information. Because volatile scent profiles are so complex and are affected by such a wide range of genetic and non-genetic factors, the same volatile profile is extremely likely to come from the same individual source. However, if a familiar volatile signal changes due to a change in the individual's status, bacterial flora or food source for example, the induction of close contact investigation by the now less familiar volatile profile will allow animals to update the link between a highly stable involatile ownership signal and the animal's current volatile profile. Fresh scent marks deposited around an animal's territory would provide ample opportunity to continually update this association in advance of encountering the owner, so that animals could use volatile profiles alone to recognise familiar conspecifics at a distance, without the need for direct contact. Thus, animals may gain the advantages of a quickly detected airborne signal that can be used to recognise and discriminate between highly familiar individuals, together with the stability and specificity of an involatile signal that will reliably indicate individual identity whenever volatile signals are ambiguous or unfamiliar.

Further, if the involatile MUP identity signal is largely responsible for the binding and release of volatile metabolites influenced by a wide range of genes as well as more specific pheromones in the scent signal (as in the MUP carrier hypothesis discussed above), this would pro-

vide a close physical link between the complex volatile and involatile profile of the individual. Although some closely related individuals are likely to inherit the same MHC type or MUP pattern, these two highly polymorphic gene complexes are inherited independently and their combinatorial diversity will be immense, making it very unlikely that even closely related individuals would share the same combination of MUPs and MHC in outbred populations.

A recent study by Hurst *et al*. (2005) used a functional bioassay to assess the respective roles of the MHC and MUPs in scent owner recognition. Male mice regularly scent mark their territories and countermark the scents of other males, behaviour which minimises aggressive encounters with competitors and increases their attractiveness to females. The experiment examined the response of males towards urine which varied in the level of genetic similarity to the territory owner, sharing either the MHC, genetic background, both MHC and genetic background, or completely disparate genotypes. Male urine derived from animals of a different genetic background to the territory owner always stimulated increased scent marking as compared with the response to own. In contrast, urine from animals which differed only at the MHC failed to raise the countermarking response. Males did increase their investigation of urine from males which differed to them at the MHC, but — in accordance with the predictions of the integrated model — *only* when this matched both the MHC and background of a familiar animal. Furthermore, having increased their level of investigation in response to odours of a different MHC type, they nevertheless failed to recognize these as different to self. These data suggest that MHC odours alone are insufficient to allow recognition of individual scent owners, although they support the hypothesis that variation in volatile odours associated with the MHC may stimulate closer investigation if they are associated with an individual of known genetic background.

Conclusions and future directions

While animals might often benefit from recognizing individual conspecifics, the mechanism by which they do so will depend on the advantages of being recognized. If there is no advantage, recognition is likely to be based on incidental cues which serve other primary functions. When animals benefit from advertising their individual identity, and in the absence of reliable and easily recognised incidental cues, selective pressure may lead to an evolved individual identity signal that allows easy and unambiguous recognition. Genetically determined signals that are expressed independently of metabolic variation have the advantages of temporal stability, combinatorial diversity and clarity over other background information. Such signals are particularly likely to be found in scents that animals deposit in the environment to provide information about themselves to conspecifics even when the owner (cue-bearer) is absent, as scent marks can only provide information about a specific depositor if they contain reliable information about the owner's identity. Two candidate systems have been proposed to carry a genetically coded individual identity signal in mice: MUPs and MHC. Both involve a set of proteins coded by a multigene complex resulting in a very high degree of combinatorial diversity, making them ideal for uniquely labelling a large number of individuals. MUPs bind and slowly release small molecular weight volatiles in mouse urine, while involatile MUP-ligand complexes are also detected directly through contact with the urine, providing signals that clearly have evolved specifically for scent communication. Initial behavioural evidence has confirmed that MUP polymorphism is involved in individual recognition, though pregnancy block responses suggest that MUPs may not be essential for recognition of familiar individuals. MHC also influences urinary odours in mice and many other species, although a specific mechanism of MHC odour expression has yet to be elucidated. If MHC influences the owner's metabolic profile, which is also influenced by non-genetic factors, this is likely to provide an unstable cue over the longer term. This might explain why MHC odour discrimination becomes much more difficult or impossible when both the genetic and environmental background are not held constant. There is ample evidence for the ability to discriminate MHC-associated odours and for a role in recognition of

kin or those expressing familiar, imprinted MHC types, but at present the role of MHC in individual recognition remains uncertain due to the lack of functional tests in this context. This is not to suggest that MHC is not involved in individual recognition. Indeed, MUPs and MHC may interact to produce individual-specific odours. If MHC plays a role through its haplotype-specific influence on individual development and physiology (Iványi 1978), these changes could in turn influence the volatile metabolites which are then bound and released by MUPs (Beynon & Hurst 2004). Further, reference to a stable involatile individual identity signal — such as that provided by MUPs — may allow animals to learn the volatile scents associated with familiar individuals so that highly familiar animals can be recognised through more easily detected incidental cues, even though these are unlikely to be stable throughout an individual's life. The ability to refer to a stable MUP identity signal when other incidental cues are ambiguous would overcome any problems with the stability of such incidental cues (Hurst & Beynon 2004).

A great deal of work to date has demonstrated that even subtle non-genetic and genetic changes, including single amino-acid substitutions in MHC peptides, produce detectable changes in the odour profile. Of these many detectable odour changes, which are relevant for individual recognition? In order to answer this question, novel empirical approaches specifically testing function, rather than discrimination ability, are required. Tests of function should also take into account the natural complexity of social odours to which animals are exposed in the wild. A particular scent cue may be used to discriminate individuals when no other information is available, such as when the cue comes from highly inbred rodents maintained on identical diets. But is this cue obscured in the face of natural background variation in odours influenced by diet, social status and health? If so, it is unlikely to be the primary basis for individual recognition.

New experimental paradigms must also measure the ability to identify a unique individual, rather than recognition based on cues which describe more than one animal (Halpin 1986, Sayigh *et al*. 1999). Experience to date has demonstrated that it is surprisingly difficult to perfect empirical approaches which test true individual recognition while eliminating the effects of familiarity and kinship. This confusion of familiarity, kinship and individual recognition incidentally raises the question of whether most animals actually have or require the ability to identify equivalent individuals. In non-social species, for example, decisions related to competition and mate choice do not require the potential competitor or mating partner to be individually identified. Competitors need to be ranked in their ability relative to self, whereas mates need to be assessed on the basis of quality and relatedness to self. It is difficult to see how such choices would be facilitated by either the advertisement or the recognition of individuality. The cases where individual recognition might really be of advantage are those in which animals repeatedly meet and interact with the same individuals, namely social species such as house mice and brown rats. Thus despite the inherent appeal of the idea that individual recognition is widespread in animals, this ability may in reality be quite restricted. Future research needs to focus on a much broader range of species than the laboratory mouse and rat, both to understand how widespread the ability to recognise individuals is and to what extent the mechanism of recognition is species specific or common across species.

It is now many years since Halpin (1986) reviewed the field of individual recognition by scent. Much progress has been made in establishing the extreme sensitivity of many rodents to small genetic and non-genetic changes in social odours, suggesting that a wealth of information is carried in these scents. However a number of the difficulties identified in Halpin's paper have yet to be overcome. In particular, the focus on the detectability of odour differences rather than function has remained in many studies, and there is still a widespread reliance on investigation as a measure of odour significance, even though this relates to perception rather than action. Nevertheless, these approaches have identified the range of social scents that are detectable by a number of rodent species, giving us a valuable starting point from which to search for those scents that function in individual recognition in the wild.

Acknowledgements

We are grateful to the Biotechnology and Biological Sciences Research Council for the support of our research. Thanks to P. T. Starks and three referees for helpful comments.

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Appendix 1. A summary of major findings obtained using the operant conditioning paradigm, with mouse and rat subjects.

Test	Subject species[1]	Donor species[2]	Odour source[3]	Donor type	Outcome	Reference
Effect of diet	R ♂	M ♂	U	MHC congenic	MHC congenic strains discriminated only when diet is also different; learned discrimination to diet cues transferred to donors of different strain	Brown *et al.* 1996
	R ♂	?R	U	MHC congenic		Schellinck *et al.* 1997
Discrimination of outbred individuals	R ♂	?R	A	Outbred strain	Two individuals of outbred strain discriminated	Schellinck *et al.* 1991
Sensitivity to changes at multiple MHC loci in inbred strains	M ♀	M ♂	U	MHC congenic	MHC congenic strains discriminated; both whole animal and urine discriminated	Penn & Potts 1998
	?M	?R	A			Beauchamp *et al.* 1985
	M ♂♀	M ♂	U			Yamaguchi *et al.* 1981
	R ♂	?R	U			Schellinck *et al.* 1997
Sensitivity to MHC on random background	M ♂♀	M ♂♀	?	F2 segregants from crossed MHC congenic	Can discriminate MHC-homozygous F2 segregants, as well as F2 homozygotes from heterozygotes	Yamaguchi *et al.* 1981
	M ♂♀	M ♂	?			Yamazaki *et al.* 1994
Detection of presence or absence of class I MHC products	M ♂♀	M ♀	U	Inbred strains; knockout strain not expressing class I MHC; F1 heterozygotes; knockout females mated with normal males	Knockouts discriminated from parental strain; heterozygotes from knockouts but not from parental strain; knockout females mated with parental strain males had detectable urinary MHC at 9–12 days gestation; knockout rat urine discriminable	Bard *et al.* 2000
	R	?R	?			Brown *et al.* 1987
Detection of change at single MHC class I gene	?	?	?	Single MHC locus mutant/congenic	Mice successfully discriminated between mice differing by between one and three amino acids in proteins coded for at a single MHC class I locus	Yamazaki *et al.* 1982, 1983, 1990
	M ♂	R ♂	U			Beauchamp *et al.* 1990
	R ♂	R ♂	U			Schellinck *et al.* 1991
Detection of change at single MHC class II gene	?	?	U	Strains congenic at a single MHC class II locus	Strains differing at a single MHC class II locus were successfully discriminated	Yamazaki *et al.* 1990
Detectability of single MHC class I locus *vs.* all 3 class I loci	R ♂	M ♂	U	MHC congenic	Equally difficult to discriminate strains differing at 3 loci as compared with strains differing at 1 locus	Brown *et al.* 1996
Expression of MHC in infants	M ♂♀	M ♂♀	A/U	MHC congenic	Whole animals not discriminated at ages 4 or 11 days; however urine from animals at age 1 day discriminated	Yamazaki *et al.* 1992a

continued

Appendix 1. Continued.

Test	Subject species[1]	Donor species[2]	Odour source[3]	Donor type	Outcome	Reference
Expression of MHC as foetus	?	?	U	MHC congenic	Paternally-derived foetal MHC types are discriminated from day 9 of gestation	Beauchamp *et al.* 1994
Genetically identical individuals	R ♂	?R	U	Inbred strains	Individuals of same inbred strain discriminated	Schellinck *et al.* 1991
Whole animal odour	R ♂	R ♂	A	Inbred strain	Rats can discriminate individuals, and subsequently on the basis bedding odour alone	Gheusi *et al.* 1997
Role of bacterial infection	M ♂♀	M ♂	U	MHC congenic (differing at either single or multiple class I loci)	Germfree MHC congenic mouse strains discriminated; individuals trained on non-germfree MHC congenic mouse strains transferred this discrimination ability to urine from germfree MHC congenics; in rats, removal of Gram-positive or Gram-negative bacteria alone alters individual odour but insufficient to eliminate individuality odours; germfree individual rats more difficult to discriminate than conventionally-housed	Yamazaki *et al.* 1990, Yamazaki *et al.* 1992b
	R ♂	R ♂	U			Schellinck *et al.* 1991
	R ♂	R ♂				Schellinck *et al.* 2000
	R ♂	?M	U			Schellinck & Brown 1992
Mouse mammary tumour virus (MMTV)	M ♀	M ♂♀	U/A	Inbred strain; F1 segregants from heterozygous transgenic with endogenous MMTV provirus	Discriminated between uninfected samples and those with asymptomatic MMTV, whether virally or genetically acquired; both whole animals and urine alone discriminated	Yamazaki *et al.* 2002
Sex chromosomes	?	M	U	Y chromosome variant inbred strains	Two strains with different Y chromosomes can be discriminated	Schellinck *et al.* 1993 Monahan *et al.* 1993
Components of urine	?M	M ♂	U	MHC congenic	Volatile acids necessary and sufficient for MHC discrimination Class I molecules purified from urine not discriminated; remaining fraction of urine discriminated	Singer *et al.* 1997
	R	R	U			Brown *et al.* 1987
MHC associated odours in blood				MHC congenic	Mice unable to discriminate untreated serum from congenic strains, but can discriminate serum treated with Pronase to liberate bound odorants	Brown *et al.* 1987
	M ♂	M ♂	U			Yamazaki *et al.* 1999

[1] The species of subject animal; R = *Rattus norvegicus*; M= *Mus domesticus*; ? = not stated (or ambiguous).
[2] The species of donor animal, codes as for subject column.
[3] Type of odour used in the trial: U = urine; A = whole animal used as an odour source.

Appendix 2. A summary of major findings obtained using the habituation–dishabituation paradigm, with house mouse and brown rat subjects.

Test	Subject	Donor	Pooled[1]	Donor type	Outcome	Reference
Effect of diet	R ♂	M ♂	N	Inbred strain	Rats dishabituated to genetically identical individuals maintained on different diets	Schellinck *et al.* 1992
Role of bacterial infection	R ♂	M ♂	N	MHC congenic strains	Germfree MHC congenic strains not discriminated	Schellinck *et al.* 1995
Discrimination of MHC congenic strains	R ♂	M ♂	N	MHC congenic strains	MHC congenic strains discriminated	Schellinck *et al.* 1995
	R ♂	R ♂	N	MHC congenic strains	MHC congenic strains discriminated	Brown *et al.* 1990
Genetically identical individuals (within strain)	R ♂	M ♂	N	Inbred strains	No dishabituation to individuals within strain	Schellinck *et al.* 1995, Schellinck *et al.* 1992
	R ♂	R ♂	N	Inbred strains	Dishabituation to individuals within strains for 1 donor strain with one subject strain only; no dishabituation for 2 other donor strains with either of 2 subject strains	Brown *et al.* 1990
	M ♀	M ♂	Y/N	Inbred strains	Dishabituation to individuals within strain, dishabituation to pools within strain	Penn & Potts 1998
Multiple samples from single individual	R ♂	R ♂	N	Inbred strain	Separate samples from single individual not discriminated[2]	Brown *et al.* 1990
Single gene knockout	M ♀	M ♂	Y	Single MHC class I gene knockout strain	Dishabituated more to single gene knockout strains than control (within strain)	Penn & Potts 1998
Single MHC class I locus	R ♂	R ♂	N	MHC class I congenic	Discriminated strains differing at single MHC class I locus	Brown *et al.* 1989, 1990
Single locus mutant on random background	M ♀	M ♂	Y	Two single locus mutant strains and parental strain; F2 segregants from 3-way backcrosses to control for background	Mutant strains discriminated from each other and from parental strain; F2 segregants discriminated from each other but not from parental strain	Carroll *et al.* 2002
Recombinant MHC class I	R ♂	R ♂	N	Inbred rats injected intravenously with recombinant class I MHC molecules	Urine of subjects injected with recombinant congenic MHC molecules discriminated from those injected with recombinant syngenic molecules	Janssen *et al.* 2001

[1] Urine from multiple individuals pooled for presentation (Y) or urine from single animals used without pooling (N).
[2] No control test reported.