# Investigating the Vibrational Hypothesis of Olfaction

Dr Simon Benedict Carleton Gane

UCL

*Supervisors*

Dr A Olaya-Castro

Prof V Lund

Prof M Stoneham

Dr M Horton

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I, Simon Benedict Carleton Gane, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Candidate's signature

## *Acknowledgements*

This thesis is dedicated to the memory of Marshall Stoneham, without whom it would not exist. I wish you could have seen what happened next. I miss your mind and our regular lunch conversations still.

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# Thesis Abstract


## *Introduction*

The sense of smell arises from the direct stimulation of olfactory receptors by chemical odourants in the nose. The exact process by which odourants trigger their specific olfactory receptors is still debated. As G protein linked receptors (GCPRs) they may be expected to have similar activation processes to those which have been identified for other similar class A receptors: induced conformational fit, but there are several reasons to suspect that the activation step of these receptors may rely on a unique, quantum level process which probes the vibrational qualities of the bonds within the olfactant molecules.

## *Hypothesis*

The olfactory receptor uses a molecular vibration-detecting method to detect the identity of its ligands. Altering the vibrational characteristics of a molecule while preserving its three-dimensional structure alters the perceived odour characteristics to humans through a receptor-level phenomenon.

## *Aims*

The aim of this thesis is to demonstrate role of molecular vibration detection in human olfaction by demonstrating a change of odour quality perceived by humans in molecules which have undergone isotopic substitution of hydrogen with deuterium, a process which affects the vibrational, but not shape, characteristics of a molecule. Once this alteration of equality was demonstrated I attempted to identify a possible responsible

receptor to facilitate further investigation of whether this is indeed a receptor-level phenomenon.

## *Objectives*

The objectives were to:

A. Demonstrate the altered odour of isotopically substituted common odourants to humans

B. Identify a putative causative olfactory receptor responsible for detection of the molecule identified in A

## *Methods*

The demonstration of an objective and reproducible alteration of molecular smell character to humans requires a psychophysical, in-vivo approach, but the locating that alteration at the level of the olfactory receptor requires examining the genes. I thus pursued two strands of inquiry: psychophysical and genomic. My methods can be summarised as:

A. Produce "probe molecule " pairs with altered vibration but not shape by selecting and deuterating the appropriate odorant (toluene, acetophenone and Exaltone)

B. Demonstrate an objective alteration of the odour after deuteration in multiple volunteers, controlling for contamination of samples, possible confounding odours, and olfactory ability; in a fully blinded experiment

C. Identify an inherited specific anosmia for a characterised molecule in a proband family

D. Phenotype the inherited specific anosmia

E. Examine the genotype via Sanger sequencing of a known candidate gene for abnormalities which segregate with the phenotype

F. Examine the genotype via exome sequencing for abnormalities which segregate with the phenotype

## *Results*

Broadly, my results demonstrate:

A. For one odorant, a musk: Exaltone, isotopic substitution alters the odour character such that human subjects are able to reliably identify the deuterated from undeuterated compound in a blinded two-element forced choice test. (Objective A)

B. This finding was not true for another odourant, acetophenone.

C. A specific anosmia for a different musk, Galaxolide, existed in three families with musk-anosmic members. (Objective B)

D. The only identified human musk receptor (OR5AN1) was not responsible for their specific anosmia to the musk Galaxolide. (Objective B)

E. I identified a new previously unsuspected possible causative variant gene OR6B3 which may be responsible for their specific anosmia and a target for further research. (Objective B)

## *Discussion*

This thesis presents three major findings. Firstly, that for certain odourants, the isotopic substitution of deuterium for hydrogen, which changes the vibrational characteristics with minimal impact on the conformation, alters the odour character such that human subjects are able to reliably identify the deuterated from undeuterated compound in a blinded two-element forced choice test. This is not true for some other odourants.

Secondly that the perception of a musk Galaxolide is impaired in three families without affecting the rest of their olfactory sense and that this specific anosmia is not caused by abnormalities in the only identified human musk receptor OR5AN1

Finally, I also identified a new previously unsuspected possible causative variant gene OR6B3.

This project is the first objective demonstration of an isotopic variation in odour quality in humans, and although I was unable to demonstrate that this was a receptor-level property, it remains a finding that is difficult to explain by the standard mechanism of olfaction and which begs further research. Especially as it is potentially a novel method of GCPR activation which may apply to other G-Protein linked Receptors.

## Impact statement

This thesis provides tentative support for a controversial theory of human olfaction: the “vibrational hypothesis”. This hypothesis posits a unique method of olfactory receptor activation reliant on quantum level effects.

These findings have potential impact far beyond the design of novel odourants, for two reasons: the ubiquity of so-called 'olfactory receptors' throughout the body and the fact that these receptors are part of a much wider family of receptors, the G-protein coupled receptors.

Recent evidence has highlighted the fact that instead of a purely 'olfactory' role for the olfactory receptors, they are found throughout the human organism and seem to play a more general chemical recognition role in tissues such as the kidney and spermatozoa. Understanding the specifics of the activation of just these receptors would enable more powerful small molecule drug design targeting these tissues.

Of potentially greater impact is the fact that this mechanism could be present in all GCPRs, which are estimated to be the target for 30% to 60% of current pharmacological agents. These quantum level effects may be responsible for the phenomenon of selective activation in which different ligands acting on the same receptor produce different pathway effects. Understanding the quantum level interactions between ligand and receptor could improve drug design and targeting.

# Publications and Abstracts

## Publications

- Gane S. What we do not know about olfaction. Part 1: from nostril to receptor. Rhinology 2010;48:131–8. doi:10.4193/Rhin09.068
- Gane S, Georganakis D, Maniati K, et al. Molecular Vibration-Sensing Component in Human Olfaction. PLoS ONE 2013;8:e55780. doi:10.1371/journal.pone.0055780
- Turin L, Gane S, Georganakis D, et al. Plausibility of the vibrational theory of olfaction. Proc Natl Acad Sci U S A 2015;112:E3154–4. doi:10.1073/pnas.1508035112

## Lectures

- "Adventures In Quantum Olfaction"- British Society of Perfumers 13 Feb 14
- "Scientific Suicide" - Royal Society of Medicine, Laryngology and Otology section 6 Dec 2014
- "Scientific Suicide - further adventures in quantum olfaction" – UK Semiochemistry Network 31 July 2013
- "Quantum Olfaction" - Wellcome trust science soirée 14 May 2013
- "How The Nose Knows A Rose Is A Rose" - RNTNEH grand round 9 March 2012
- "Quantum Smell" - SMARTS (St Mary's Alumni Research and Teaching Society ) 2011
- "A Bad Smell - A literary mystery" - St Mary's Grand Rounds 29 Oct 10
- Psychophysics of the Swipe-card model" - UK Semiochemistry Network British Workshop XVIII 9-10th September 2010
- "The human olfactory receptor" - RNTNEH Grand Round 5 May 2010
- "The human olfactory receptor" - UK Semiochemistry Network British Workshop XVII 16th September 2009
- "Investigating a Novel Theory of Olfaction" – Otolaryngology Research Society 25 Sept 2009
- What we (don't) know about olfaction - UCL Ear institute research day 21 Oct 2008

**Poster Presentations**

- UCL Neuroscience Symposium Jun 2013 - Molecular vibration sensing in human olfaction: Gane, Olaya-Castro, Lund
- A celebration of disordered matter: hard spheres, wet molecules, and awkward ice, Jan 2009 – "It pays to be flexible. The underestimated role of flexibility in small biomolecules" - Brookes, Gane, Stoneham
- STINKFEST 2008 - "That Stinks! Does your nose use quantum physics to smell the sewer?" Simon Gane, Jenny Brookes, Andrew Horsfield, Marshall Stoneham.

# Glossary and list of abbreviations

- AC III - Adenylyl cyclase III catalyses the formation of cAMP from ATP
- CNG - cAMP activated cationic channel in OSN membrane
- C-P450 – cytochrome P450 a family of heme enzyems responsible for multiple enzymatic reactions in biological systems.
- Glomerulus - Functional unit of the olfactory bulb that segregates and organizes the synaptic inputs from the OSNs with their partner output neurons (the mitral cells), which in turn carry information from the olfactory bulb to higher cortical centres.
- $G_{olf}$ - G-Protein associated with olfactory receptors
- GPCR - G protein–coupled receptor; representing a large superfamily of receptors with a characteristic seven-transmembrane topology and whose intracellular signals are transduced by heterotrimeric G proteins; includes receptors for hormones, neurotransmitters, visual stimuli, and chemosensory (olfaction and taste) stimuli.
- Headspace - a perfumery term for the amount of air in a container of odorant into which that odorant must diffuse in order to be smelled.
- Homolog - A gene related to a second gene by descent from a common ancestral DNA sequence. The term, homolog, may apply to the relationship between genes separated by the event of speciation (see ortholog) or to the relationship betwen genes separated by the event of genetic duplication (see paralog).
- OBP - Odorant binding Protein

- Odo(u)r - Scent or smell; natural sources of odours are often complex mixtures of many compounds, some of which (but not necessarily all) can contribute to the perception of the particular smell.

- Odorant - A compound that elicits the perception of smell.

- Odorant receptors - Receptors expressed by OSNs and belonging to the GPCR superfamily, responsible primarily for receiving “chemosensory” or chemical cues from the environment. There are multiple families of odorant receptors, which include the OR (the largest family), TAAR, V1R, V2R, and formyl peptide-like receptors.

- Olfaction - The sense of smell.

- Olfactory bulb - A specialized structure of the forebrain, which receives direct input from the OSNs in the nose.

- Olfactory Binding Protein – secreted protein of the lipocalin family which binds small molecules within the olfactory mucus

- Olfactory cortex - Collectively refers to the five brain regions that receive direct input from the olfactory bulb: the piriform cortex, anterior olfactory nucleus, olfactory tubercle, entorhinal cortex, and amygdala. The regions of the olfactory cortex are responsible for the perception of smell and for generating odor-evoked behaviors.

- Olfactory epithelium - The sensory epithelium that resides in the nose and contains the primary sensory neurons (the OSNs) that are responsible for detecting chemical stimuli from the environment.

- Olfactory sensory neuron - The primary sensory neuron of the olfactory system, responsible for receiving and transducing chemical information from the environment. The olfactory sensory neuron is where the olfactory system meets the outside chemical world.
- OBP - Olfactory Binding Protein
- OR - Olfactory Receptor
- ORN – Olfactory Receptor Neuron (also called olfactory sensory neuron)
- Ortholog - Orthologs are genes in different species that evolved from a common ancestral gene by speciation. Normally, orthologs retain the same function in the course of evolution. Identification of orthologs is critical for reliable prediction of gene function in newly sequenced genomes. (See also Paralogs.).
- OSN - Olfactory Sensory Neuron (also called Olfactory receptor neuron: ORN)
- Paralog - Paralogs are genes related by duplication within a genome. Orthologs retain the same function in the course of evolution, whereas paralogs evolve new functions, even if these are related to the original one.
- Projection neuron - The mitral or tufted cell in the case of the olfactory bulb; these neurons receive information from the OSNs in the olfactory epithelium, and relay or "project" this information to the next level in the olfactory cortex.
- QSAR - Quantitative Structure-Activity Relationship
- SNP - single nucleotide polymorphism

- STV - Specific Threshold Variant - a hypo-, hyper- or an-osmia to a specific odorant with a well-preserved general olfactory ability

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# Chapter One: Literature Review

## Chapter Synopsis

In this chapter, I set out the history and current understanding of the vibrational hypothesis of olfaction. I summarise the initial statements of the theory and its historical development. I then critically assess the evidence base in vertebrate and invertebrate animals as well as in theoretical modelling. This is followed by an overview of the olfactory system, with special attention paid to the possible sources of odour alteration, the genetics of the olfactory receptor and including the what is currently known about other non-olfactory receptor G-Protein coupled receptor activation.

I conclude this chapter with the aim and objectives of my thesis and an overview of its structure.

## The Vibrational Hypothesis of Olfaction

*"Olfactory theories are as numerous as pebbles on a beach"*

- JT Davies (Davies 1971)

One of the central problems of olfaction has long been understanding the mechanism by which an inhaled molecule becomes a smell percept. This exact "how" of olfaction has been debated since antiquity.

Ancient Greek atomists are said to have connected the smooth shape of certain atoms with their sweet smell (Sell 2014) Even as late as the 1960s Roderick (Roderick 1966) collated about thirty different hypotheses as to the exact nature of what makes an odorant smell the way it does. That the olfactory sense was receptor-based was widely assumed for

several decades before the discovery of the olfactory receptors (ORs) by Buck and Axel in the early 1990s, but even once they found the olfactory receptors, arguments raged about how exactly these detected the odorants.

TABLE I
PROPOSALS REGARDING THE PHYSIOCOCHEMICAL BASIS OF OLFACTION

| Author | Date | General class | Salient features |
|---|---|---|---|
| Ogle | 1870 | Vibrational | Vibrations affected nasal pigment, which gave out heat that excited the olfactory cells |
| Woker | 1906 | Chemical | Unsaturation main cause of odor, but not essential if substance very volatile |
| Fabre | 1911 | Vibrational | Limited to insets. Not known by man. Human olfaction due to material particles |
| Marchand | 1915 | Chemical | Unsaturation (including >C=O). Two points of unsaturation reduces odor |
| Henning | 1916 | Chemical | Osmophore groups are important, but their relative position determines the type of odor |
| Heyninx | 1917 | Vibrational | Vibrations causing absorption in the ultraviolet band also caused odor |
| Backman | 1917 | Chemical | Water solubility and lipid solubility essential |
| Teudt | 1919 | Vibrational | Electronic vibrations of sensory nerves increased by resonance with similar vibrations of odorants |
| Durrans | 1920 | Chemical | Residual affinity. Addition reaction on the olfactory epithelium |
| Heller | 1920 | Chemical | Direct chemical action on nerve-ending |
| Ruzicka | 1920 | Chemical | Osmophore and osmoceptor |
| Tschirch | 1921 | Chemical | Substance must be soluble in air. Loose compound formed with plasma of the olfactory cell |
| Zwaardemaker | 1922 | Chemical-vibrational | Possess odoriphore, be volatile, lower surface tension, lipoid soluble. Odoriphore depends on vibrations in molecule |
| Ungerer and Stoddard | 1922 | Vibrational | Intramolecular vibrations within definite frequency range. Unsaturation helpful. Interference and resonance effects |
| Delange | 1922 | Chemical | Unsaturation |
| Missenden | 1926 | Chemical | Intensity depends on number of molecules making contact with nose. Quality depends on nature of reaction between odorous molecules and lipoid tissues |
| Nicol | 1926 | — | Function of sinuses |
| Pirrone | 1929 | Chemical | Two osmophore groups, one determines type of odor, the other the variety |
| Niccolini | 1933 | Chemical | Volatility. Solubility in nasal mucosa. Oxidizability |
| Krisch | 1934 | Vibrational | Insects |
| Müller | 1936 | Physical | Odorous substances are dipolar. Irritate the molecular fields of the osmoceptor in nose |
| Dyson | 1937 | Vibrational | Volatility. Lipoid solubility. Raman shift between 1400 and 3500 $cm^{-1}$ |
| Beck and Miles | 1947 | Vibrational | Infrared radiation from receptors absorbed by odorants |

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*Figure 1:"Historical theories of olfaction" Table 1 from Cain (Cain 1978)*

As is shown in Figure 1, there are have been many theories promulgated about the recognition step in olfaction such as the 'Profile-Functional Group' theory suggested by Beets (Beets 1957) in 1957: that the smell of a substance relied on two things: primarily the functional group (e.g. all –SH bearing molecules smell sulphuraceous) and secondarily the overall shape or form.

Steric theory, also known as "lock and key" has remained the quintessential model for ligand recognition of all types. First proposed for smell by Moncrieff (Moncrieff 1949), it was elaborated as the "stereochemical" theory by Amoore in the early 1960s (Amoore 1963). He proposed seven "primary odours" analogous to primary colours, and a receptor for each. The initial one receptor–one smell theory has gone by the wayside but there is no doubt that allosteric interactions must have some role in receptor function.

Most other hypotheses apart from the steric or "induced conformational fit" model have fallen away but one competing theory remains: the vibrational hypothesis. I summarise and then expand on the literature around the vibrational hypothesis below, demonstrating how it leads to the experiments reported in this thesis.

## Summary of the Literature on the Vibrational Hypothesis

The literature for and against the vibrational hypothesis is contradictory and of varying quality. The initial Turin hypothesis (Turin 1996) has several internal inconsistencies, including an inability to account for the three-dimensional shape and individual receptor interactions. This is improved with the "swipe card model" which he helped to develop (Horsfield 2012). Human experiments such as those performed by Keller and Vosshall (Keller & Vosshall 2004) did not support several of the claims of the initial paper, although those performed by Haffenden (Haffenden et al. 2001) did. Both papers have some flaws

in their methods. Insect experiments are much more convincing but are also conflicting. The clearest demonstration of insect ability to detect deuteration through a vibration-detection mechanism was the work of Franco et al (Franco, Turin, Mershin & Skoulakis 2011b), which has been partially replicated.

Theoretical work is necessarily more limited without the structure for any olfactory receptor, but although earlier models of the mechanism were promising (Brookes et al. 2007), more recent work with better estimates of the constants involved throws doubt on the possibility of a quantum level molecular detection mechanism (Block, Jang, Matsunami, Sekharan, et al. 2015). One explanation for this might be that vibrational modes are a good summary of the underlying molecular structure which is required to activate the receptor, but not directly detected themselves (Gabler et al. 2013). Importantly, there is support for the fact that deuteration of a molecule alters its suite of molecular vibrations, which is predicted to alter its smell if the vibrational hypothesis holds true (Block, Jang, Matsunami, Sekharan, et al. 2015).

The literature lacks the definitive demonstration of a central prediction of the vibrational hypothesis: that altering the vibrational modes of a molecule while maintaining the surface topology or "shape" alters the odour quality and that this is a property, not of the system as a whole, but of the interactions with the receptor.

Providing this evidence, or refuting it, is the central aim of this thesis.

**The "Undulatory Hypothesis" – Early Versions of the Vibrational Hypothesis.**

Dr Ogle in 1870 (Ogle 1870), is the first in the literature to suggest the "undulatory hypothesis" of olfaction. In discussing a case series of anosmics, he suggested the pigment of the olfactory mucosa:

*"may serve to absorb vibrations of odour and of sound, and to convert them into vibrations of heat, which will affect the contiguous cells."*

Although Ogle thought that the vibrations would be of heat, Haycraft in 1888 (Haycraft 1888) did not specify the medium, stating rather that:

*"smell, like sight and hearing, depends for its production on the vibrations of the stimulating medium, the quality of the sensation depending, in all cases, upon the kind of vibration which produces it."*

Almost fifty years later Malcolm Dyson (Dyson 1938) promulgated the "scientific basis of olfaction", noting the close correlation between the Raman spectrum of a molecule between 1400 and 3500 wavenumbers and its odour. He notes that Faraday had noticed that odorous substances absorb infrared radiation (no reference is given), but makes no mention of a possible mechanism "by which the odorous particles once in contact with the sensory processes, give rise to their characteristic odour sensation".

Even in the written record of the first presentation of the theory there was evidence of intense debate and counter-argument, which would continue to dog the theory in its iterations after.

In 1954 the Canadian RH Wright (Wright 1954), in a letter to Nature, set out the project which was to occupy him for the next few decades and which would lead to the vibrational hypothesis being referred to as the Wright hypothesis for much of this time. He noted Dyson's model but rejected the "osmic frequencies" between 1500 to 3000 cm^-1 as being too closely associated with specific functional groups -OH, -CO etc. and therefore if odour correlated with vibrations in these frequencies it would as easily be correlated with the physiochemical properties of those functional groups. Instead Wright focused on the

region below 1000 cm^-1 and reported the association of low-frequency Raman shifts with odour although he does not give the evidence. He does note that the method allowed him to find a molecule in the literature with a similar odour to a group of similar molecules, *"something no other theory of the physical basis of odour would allow one to do."* Limiting the range of detectable vibrations to below 1000 wavenumbers or even 500 excluded several powerful inorganic odorants such as ammonia and hydrogen sulphide, which only vibrate in the 700 to 3500cm^1 range (Klopping 1971).

Wright would go on to work on the vibrational hypothesis for the next twenty-five years, although the theory was never really accepted by the majority of smell researchers. This was because of the inability to account for enantiomeric odour differences, the need for a higher frequency range to account for the odour of some inorganic odorants and the lack of a suitable mechanism: how would an organism make an infra-red spectroscope?

In the late 1980s the biophysicist Luca Turin, already interested in perfumery as a hobby, came across the Wright theory. This, together with his knowledge of a new form of spectroscope, the electron-tunnelling spectroscope, led him to posit that the newly-discovered olfactory receptors might act as a kind of fixed-frequency spectroscope, with a suite of such receptors covering the spectrum of interest. (Burr 2012)

**Introduction to the Turin Hypothesis**

Published in 1996 in Chemical Senses by special acceptance of the editor and without peer review, "A spectroscopic method for primary olfactory reception" (Turin 1996) was Turin's first published public statement of his biological inelastic electron tunnelling spectroscope (IETS) theory. The paper was discursive, covering several parts of his investigations into the mechanism of activation of the olfactory receptor. Building on

Wright's vibrational theory (Wright 1977), he described a quantum mechanical mechanism of vibrational bond detection by means of inelastic electron tunnelling within the receptor.

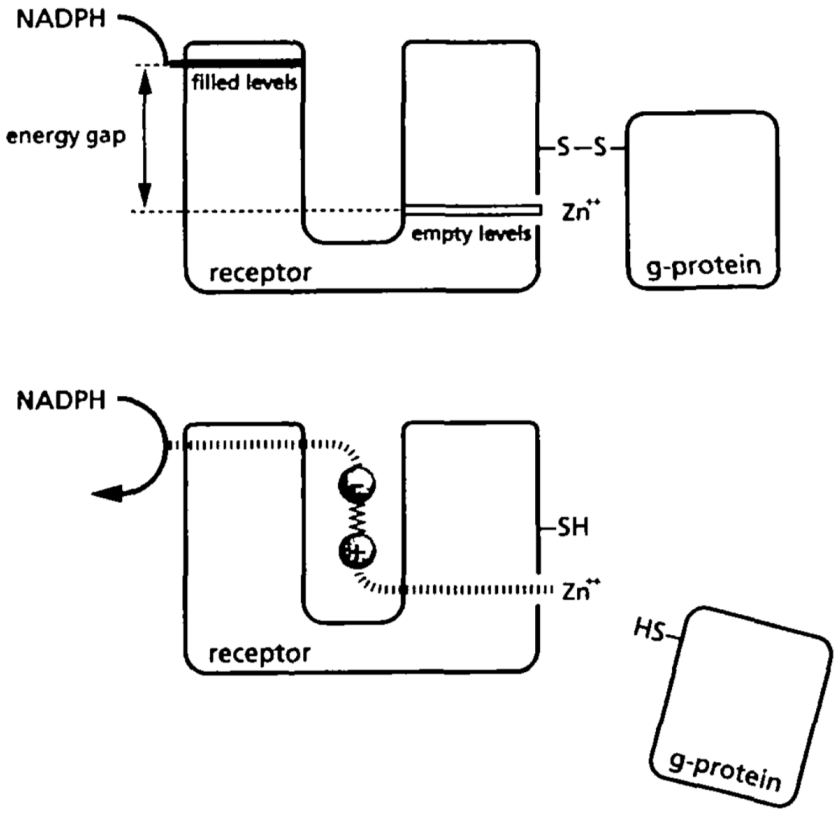



*Figure 2: Figure 1 from Turin (Turin 1996)Schematic of the proposed transduction mechanism: the receptor protein accepts electrons from a soluble electron donor (NADPH). When the receptor binding site is empty (top), electrons are unable to tunnel across the binding site because no empty levels are available at the appropriate energy. The disulphide bridge between the receptor and its associated G-protein remains in the oxidized state. When an odorant (here represented as an elastic dipole) occupies the binding site (bottom), electrons can lose energy during tunnelling by exciting its vibrational mode. This only happens if the energy of the vibrational mode equals the energy gap between the filled and empty levels. Electrons then flow through the protein and reduce the disulphide bridge via a zinc ion, thus releasing the G-protein for further transduction steps.*

In essence, the Turin hypothesis posits a fixed-frequency molecular level spectroscope built within the receptor. A simple model requires an electron donor (suggested to be NADPH) on one side with a source of electrons at a high energy level and, across the odorant binding site, an acceptor with an empty site at a lower energy. For an electron to cross to the acceptor it must be able to lose a specific amount of energy whilst

tunnelling across the gap. It is able to lose this energy to the vibration of the bonds within the molecule if they vibrate with a frequency that can accept that energy.

The quantised nature of energy at the subatomic level means that energy can only be lost in integer multiples of the Planck constant and in integer multiples of the wavelength of the odorant vibrations, in short:

*"The odorant vibrations that are far above kT at body temperature are normally unexcited and therefore in the ground state. The odorant accepts one quantum of energy for a particular mode from the passing electron which loses energy in the process and can therefore tunnel from a higher to a lower energy site."*

[Turin 2014, pers comm]

This allows the odorant to act as a gate: the electron can only move to the acceptor if there is an odorant with a valid bond vibrational frequency in the binding site. The presence of an electron on this site means that the specific vibrational frequencies that fit into the energy gap between the donor and acceptor sites must have been present. *Et voilà* - a working spectroscope, albeit for only a few, fixed wavelengths. Turin posits that there is a piecewise division of the available spectrum by overlapping receptors in analogy with the visual system.

Turin's paper did not address the possible contributions by shape, appearing to posit only a vibrational sense of smell. Further adaptions have refined the theory to what I call the "modern Turin hypothesis"

**A Unique OR Mechanism?**

Why should olfactory receptors be different?

If the vibrational hypothesis is true, it would mean a hitherto unknown mechanism of activation for a subset of GPCRs which are otherwise quite similar to others, for which we have a robust model for ligand detection and activation. Why would that be desirable from an evolutionary standpoint? The answer lies in the tuning of the receptors. Some of the closest in sequence to the olfactory receptors, such as rhodopsin, bind only one ligand (11-cis-retinal in rhodopsin's case) and most hormone receptors for instance, will require a very narrow range of possible ligands, to avoid false-positive activation.

Olfaction is different, the point is to recognise the widest range of chemical signals and act on them meaningfully. If a mechanism such as inelastic electron tunnelling were physically possible, the ability to identify possibly toxic atoms within molecules would be extremely useful, something which responding to the outer structure of a molecule would not be able to provide.

The work of Nara et al (Nara et al. 2011) on disassociated rat neurons demonstrated that there appeared to be two groups of olfactory receptor neurons: those reacting broadly to a wide range of odorants, and those which seem to be much more precisely tuned, reacting to only one or two of the tested ligands. Could it be that there were two separate mechanisms determining the ligand specificity of each of these classes of OSNs? Since we unfortunately do not know the receptor identities of these neurons we cannot examine them for structural similarities or sequence correlation between the groups.

An entirely novel activation system for a subset of ORs may be special pleading, but as Audet and Bouvier put it in a review of GPCR activation (Audet & Bouvier 2012): "The diversity in binding modes also has important implications for the activation process of the receptors, as it suggests that no unique activation trigger can be invoked for all GPCRs", and therefore a vibrational explanation is not excluded.

Without a structure for any OR, the answer remains elusive.

**Turin's Original Hypothesis**

In his 1996 paper, Turin (Turin 1996) set out the electron tunnelling vibrational hypothesis, sketching a model for a range of fixed vibration detectors within the ORs. The paper is discursive and broad ranging, covering evidence from protein structure to quantum physical modelling. His evidence is somewhat circumstantial, as might be expected in an initial statement of theory and can be divided into approximately three broad categories: structural motifs for required electron donor and acceptor sites, neurophysiological evidence, and structure-odour relations (psychophysical methods).

Turin began by examining the published primary structures for twenty-eight olfactory and eight gustatory receptors (all that were available at that time) for possible binding sites for electron donors and acceptors. He found a previously identified NAD(P)H binding site on all of the receptors with small variations although he did not give the location and whether the location is conserved. Surmising from clinical and other evidence that zinc may be the metal electron acceptor he examined the primary structure close to the flavin site identified above and found a conserved amino acid pattern C[GATV]SH[LI], commonly CGSHL, in all the ORs at the cytoplasmic end of the predicted sixth transmembrane loop. This is the zinc binding site of insulins and in a search of the swissprot database only insulins, olfactory and gustatory receptors, and three other proteins gave an exact match. He found a matching possible zinc binding site on $G_{olf}$ and suggested that it was a reversible reduction of a disulphide bridge between $G_{olf}$ and the OR via ionisation of the Zn which was the activating step in the cascade.

The neurophysiological evidence he gave is limited, as Turin was unaware at the time of the work of Clifton Meloan (vide infra), but he did discuss the findings of Firestein et al

(Firestein et al. 1993). In investigating the activity of the tiger salamander OSNs in response to three odorants they demonstrated cells which responded to one, any two or all three. Turin analysed the spectra for the odorants and showed that the results were consistent with receptors responding to three different bands of vibration, see Figure 3.

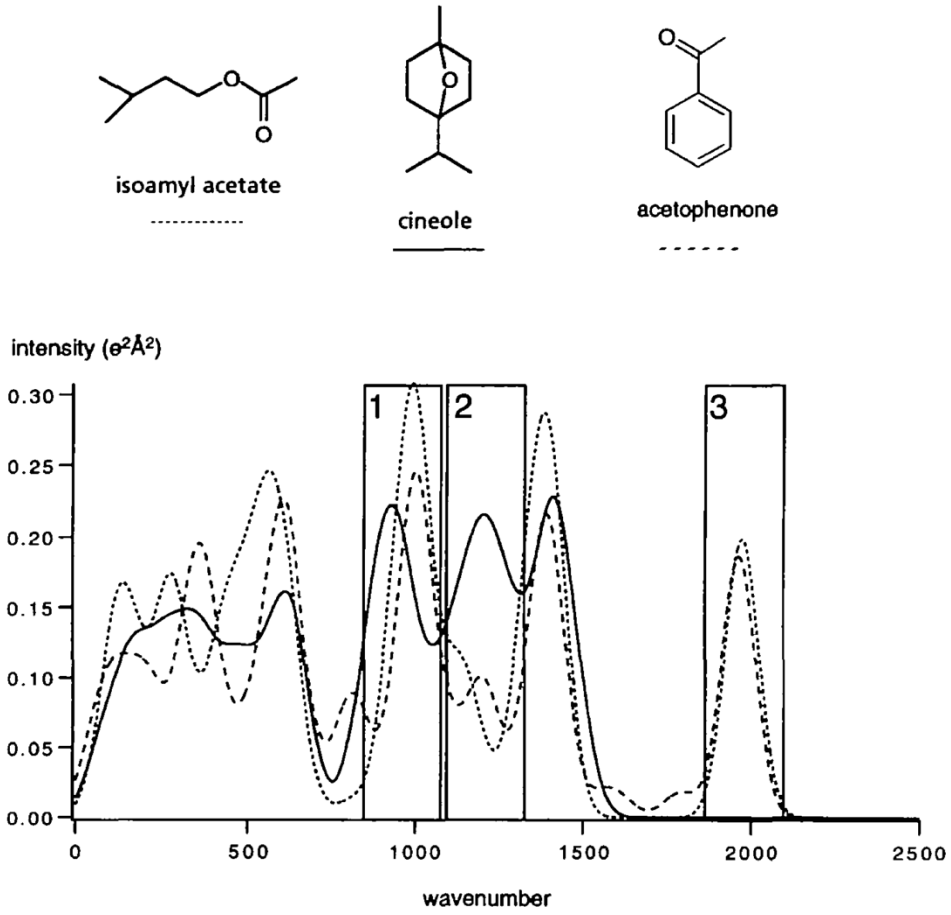



*Figure 3: Figure 4 from Turin (Turin 1996): Structures and CHYPRE spectra of three odorants used by Firestein et al (Firestein et al. 1993)*

The rest of the paper is an extended theory of structure-odour relations demonstrating the ability of the vibrational hypothesis and Turin's CHYPRE spectrum

calculation algorithm in predicting odour character. CHYPRE is a molecular modelling program modified to generate what he states is a more plausible "biological" IETS spectrum.

In these sections Turin gave a great many examples of odour character as evidence. Many of these were statements about his own perception, delivered as self-evident. Although the sources and variation in human olfaction were not as appreciated then as they are now, he did not spend much time in controlling biases in these tests. A typical statement of altered odour character is merely stated as fact: *"The acetophenone -d8 had the same odour as the bulk sample and was clearly different in odour from the acetophenone peak"*. (A finding which is examined in this thesis).

When statements of odour character or intensity of a more general type are made they are often not referenced *"Vanillin has a rich vanilla odour, whereas isovanillin has a weaker, very different, somewhat phenolic odour".* Some odour qualities are given from the standard reference "Perfume and Flavour chemicals" (Arctander 1994) and some from other papers e.g. Klopping (Klopping 1971). Turin's reliance on his own well-developed sense of smell translated into an avenue of weakness as his findings could not be replicated in larger groups such as the studies by Keller and Vosshall (Keller & Vosshall 2004).

In his quest for a unified structure-odour relation theory, Turin considered several problems. The first is the overlap in odour character between cedramber, Jeger's metal, charnel and timberol. These molecules strongly share an ambergris scent and yet are structurally very different, but when their raw vibrational spectra are convolved with a "blurring" function, to mimic the presumed lack of precision of a biological IETS system, the spectra become very similar.

He then examined the opposite case, where molecules have very similar structures and yet smell very different. The molecules he cites are 2-, 4- and 6-undecanone, which differ only in the location of the carbonyl group yet the 2- and 6- smell very different, with the 4- having an intermediate odour as discussed in Ohloff (Ohloff 1986). Once again, the CHYPRE spectra are different for the 2- and 6- undecanone, and the 4- falls somewhere between.

Turin then moved onto a discussion of the possible ways in which a molecule which might otherwise be expected to be odorous might not have a scent. He divided these up into six possible categories: the lack of partial charges, molecules larger than a maximum size, the lack of receptors covering certain vibrational bands, vibrational frequencies too low, weak scattering due to stiff bonds, and molecules where an electron scattering group scatters electrons away from the electron acceptor.

Turin used this last model as an explanation for the famous difference in the odour of carvone enantiomers, suggesting that the carbonyl group on the s–carvone scatters the electrons away from the acceptor in a way that the r–carvone does not. He quotes Weyerstahl (Weyerstahl 1994) to support this in an experiment where the carbonyl is replaced by an alcohol for both enantiomers, the minty odour is retained by the r-carvone yet the s-carvone's odour is completely altered.

It is here that Turin introduced a, now obvious, error in the hypothesis, one which is corrected in the 'modern vibrational hypothesis'. He posited that missing vibrations can be 'added back in' by a mixture with other odorants containing the missing vibrations. This would be a sensible suggestion if there were a continuous vibrational detecting sensor, such as the tonotopic map of the cochlea, i.e. in the absence of multiple specific receptors. Even within this version of the vibrational hypothesis Turin suggested multiple fixed frequency

receptors so that other vibrations will necessarily be mediated by a different receptor. He assumed that the higher-level processing will be a simple additive process, with the receptor outputs being merely added together in the brain and suggests that this 'additive synthesis' might be useful in generating a vanillin-like odour from mixing guaiacol and benzaldehyde, neither of which have a vanilla-like smell. This was investigated by Keller and Vosshall in 2004 (Keller & Vosshall 2004) (vide infra) and found to be false.

Turin's consideration of what he called 'monochromatic' odours remains one of the most powerful arguments for the vibrational hypothesis. His example is still not adequately explained by alternative hypotheses. He notes that the thiols (-SH) or mercaptans, selenomercaptans (-SeH) and telluromercaptans (-TeH) share a unique odour character, one familiar to anyone who has smelled a rotten egg. Turin found the stretch vibration frequency (~2500cm^-1) to be similarly unique, not overlapping with any other common group frequency (Socrates 2004). The only other group to have stretch frequencies which vibrate in this region (~2600 cm^-1) is the terminal boron-hydrogen (-BH) group. Turin made the statement that decaborane is the only molecule to his knowledge which smells like a mercaptan and yet does not contain sulphur. He also identified other phosphine and arsine vibrations, which lie near the 2500 cm^-1 band and which are reported to smell like garlic or rotten cabbage, two sources of complex mercaptans.

He gave several other, less thoroughly explored, examples of single bands giving rise to similar odours. These monochromatic odours however cannot explain the famous conundrum of the bitter almond odour (over 40 odorants share a similar bitter almond odour and very little in the way of structural similarity) so he constructed a self-admittedly ad hoc explanation without much in the way of explanatory power, referring to this as a bichromatic odour character.

In dealing with the aliphatic aldehydes he once again gave as evidence that the pattern of changing odour of the aldehydes with increasing chain length that it is "well known perfumers". This similarity in odour character between the even numbered aldehydes (fruity, orange-like), with the odd numbered aldehydes sharing a different (more floral or waxy) similar character is not evidenced any further and it is this prediction and two others which are shown to be unfounded by the Keller and Vosshall (Keller & Vosshall 2004) paper eight years later.

Keller and Vosshall did not assess the odour of the metallocenes, complex molecules where metal atoms are buried within cyclopentdeinyl rings, possibly because the molecules are thought to be toxic. These molecules are very similar in size and shape and have very similar IR spectra except for the vibrations of the metal-ring bond. The metals are not exposed and yet Turin stated their odours to be different. If this is a purely receptor level phenomenon it is difficult to see how a shape theory can account for this.

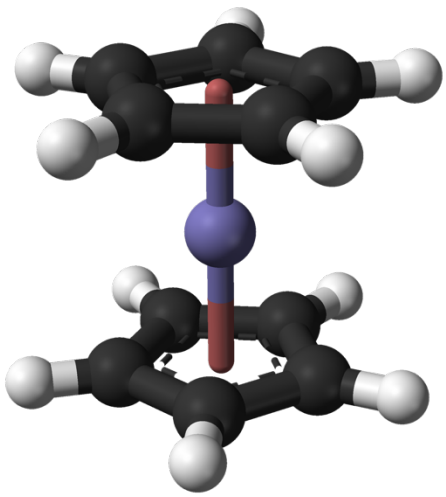


*Figure 4: "Ferrocene-from-xtal-3D-balls" by Ben Mills - Own work. Licensed under Public Domain via Commons*

The final section in this work is a description of the presumed variation in odour character and IR spectra of deuterated and undeuterated acetophenone, a claim examined in detail in this thesis.

**The Modern Turin Hypothesis**

Turin did not address the role of the three-dimensional shape of the odorant in his 1996 paper. It is difficult to find a clear statement of the opposition theory for smell but the consensus is that it operates on a similar mechanism to the pharmacological model of "induced conformational change", where the binding of the agonist causes a change in the energy landscape of the molecules such that the activated state is at a lower energy and the receptor assumes that form.

It is obvious from a simple understanding of receptor morphology that a binding pocket will only fit a limited number of odorants. The number of receptors must not only cover the range of permutations of up to 18 carbon molecules, but also the vibrational spectrum too. Turin's theory required some alteration in the light of these facts.

Also called the swipe-card model (named by Prof M Stoneham), the modern version posits that not only must the odorant possess the correct shape to bind the receptor ( as a swipe card must be thin enough to fit into the reader), it must also possess the correct bond vibrations (the correct information on the magnetic strip of the card) (Horsfield 2012). Only when both of these conditions are true will there be activation of the receptor.

**Experimental Evidence for the Vibrational Hypothesis**

Insect Experiments

The history of using insect olfaction to examine the vibrational hypothesis extends from the middle of the twentieth century. Wright (Wright 1974) in 1974 noted the difficulties in using human subjects to analyse odour and preferred insects because : "A

small insect probably has only a few genetically fixed pattern-recognition systems or templates in its tiny brain."

In the same paper, he described several bioassays of novel compounds which were selected using their spectroscopic similarity to known chemical attractants, some of which were structurally dissimilar but still effective as attractants, although he still required experimental evidence to determine which was which. Once again, the theory seemed to be strongest in its ability to find new molecules to examine rather than to predict with any power which would be functional. In the conclusion Wright argued that "one positive result signifies more than a dozen negative ones"(Wright 1974), and while this is true, it does not absolve the investigator of attempting to falsify a theory rather than confirm it.

Invertebrate Olfaction of Deuterated Compounds

Other researchers investigated what was then known as the Wright theory of olfaction using deuterated olfactants or semiochemicals in insects in several papers in the late sixties and early seventies. The results of which are summarised in Table 1.

| Year | Team | Insect | Assay | Chemical | Deuteration | Effect? |
|---|---|---|---|---|---|---|
| **1968** | Doolittle{Doolittle:1968ca} | Dacus curcubitae | behavioural (netted box) | 4-(*p*-hydrophenyl) 2-butanone acetate | several sites | no |
| **1971** | Blum{Blum:1971cy} | Pogonomyrmex badius | behavioural (alarm aggregation) | 4-methyl-3-heptanone | several sites | no |
| **1973** | Barker {Barker:1973ci} | Apis mellifera | conditioned behavioural | nitrobenzene | perdeuterated | no |
| **1978** | Sugawara {Sugawara:1978vx} {Tominaga:1981ju} | Blatella germanica L. | free field behavioural | propyl cyclohexanaceteate | perdeuterated | equivocal |
| **1981** | Wang{Wang:1981tl} | Tibolium casteneum (Herbst) | behavioural (aggregation) | acetaldehyde | perdeuterated | yes |
| **1982** | Kuo{Kuo:1982ug} | Periplaneta americana L | behavioural(aggregatin) | bornyl acetate | perdeuterated | yes |
| **1984** | Scriven{Scriven:1984tb} | Periplaneta americana L | electro-antennagram (EAG) | bornyl acetate | perdeuterated | yes |
| **1985** | Havens {Havens:1995bf} | Periplaneta americana L | electro-antennagram (EAG) | bornyl acetate | several positions | yes |
| **1995** | Decou {DeCou:1995ug} | Periplaneta americana L | electro-antennagram (EAG) | (e)-2-hexen-1-al | several sites | yes |
| **2011** | Franco{Franco:2011ij} | Drosophila melanogaster | behavioural (t-maze) | benzaldehyde, acetophenone, citronellyl nitrile, citronellyl aldehyde | partial and per-deuterated | yes |
| **2012** | Bittner{Bittner:2012fz} | Drosophila melanogaster | behavioural (T-maze) | acetophenone | perdeuterated | yes |
| **2014** | Gronenberg{Gronenberg:2014kw} | Apis mellifera | behavioural (proboscis extension) | acetophenone & benzaldehyde | perdeuterated | yes |

*Table 1: Summary of deuteration experiments in insects*

The preponderance of the evidence was that deuteration did have some effect on pheromonal or olfactory function in insects. This effect was more obvious with electrical rather than free-field behavioural assays, although controlled behavioural trials were also successful in showing alteration in effect.

" Molecular Vibration-Sensing Component In Drosophila Melanogaster Olfaction"

In 2011 Turin, working with Makis Skoulakis and others published the first evidence of a vibrational, quantum effect in insects (Franco, Turin, Mershin & Skoulakis 2011b). In a thorough investigation of *Drosophila* behaviour they exposed several hundred fruit flies to pairs of odorants presented in T-mazes, a well-recognised methodology for evaluating odour preference (Skoulakis & Grammenoudi 2006; Pavlopoulos et al. 2008). The results are very difficult to explain by any other theory of olfaction and are worth examining in detail.

In a careful set of experiments the team demonstrated four properties of *Drosophila* olfaction:

1: Drosophila spontaneously distinguish between deuterated and non-deuterated odorants

2: This discrimination could be a target of conditioning, independent of spontaneous preference.

3: The conditioned isotope response was generalisable between molecules containing the same isotopes. Drosophila trained to avoid deuterium in one molecule avoid it in other molecules.

4: Not only this but they could generalise this quality to other non-deuterated molecules of a similar vibrational spectrum.

### *1: Drosophila spontaneously distinguish between deuterated and non-deuterated odorants*

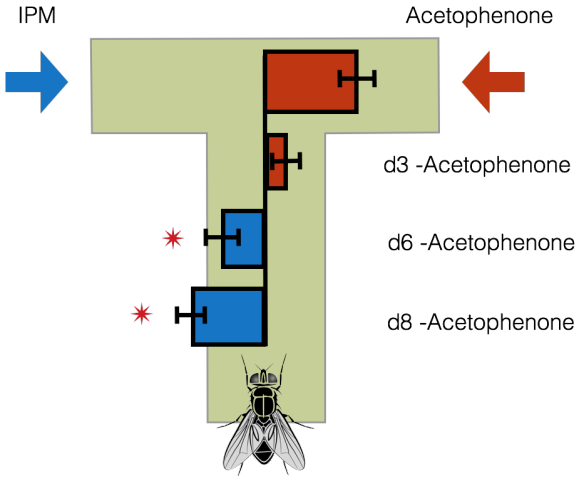



*Figure 5: Drosophila spontaneously distinguish between deuterated and non-deuterated odorants. Redrawn from Figure 1 of Franco et al (Franco, Turin, Mershin & Skoulakis 2011b) Bar length is proportional to the number of flies in the arm. * denotes p<0.05.*

Flies demonstrated an untrained avoidance of deuterated acetophenone (ACP) vs IPM in direct proportion to its degree of deuteration. They also preferred the undeuterated ACP against its d8 analogue, the concentration of which had to be halved to balance the maze (equal numbers of flies in each arm). The preference was even stronger in the case of octanol vs d8-octnanol but interestingly this aversion did not generalise to d5-benzaldehyde, which was only aversive when at double the concentration of the hydrogenated molecule. To exclude any non-olfactory cues, two anosmic mutants were tested with ACP and octanol, neither showing any asymmetric distribution in the T-mazes.

*2: This discrimination could be a target of conditioning, independent of spontaneous preference.*

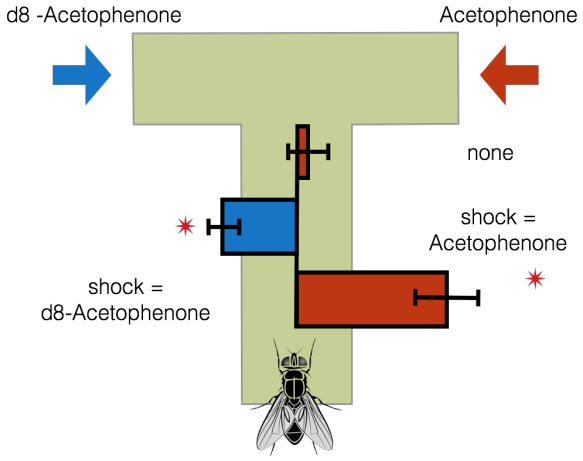



*Figure 1.6: This discrimination could be a target of conditioning, independent of spontaneous preference. Redrawn from data in Figure 2, Franco et al (Franco, Turin, Mershin & Skoulakis 2011b)*

In a balanced maze the flies were trained to associate foot-shock punishment with one or the other of the deuterated/non-deuterated pair. When released into a new maze, they preferred the arm containing the other (non-punished) stimulus. This indicates that the presence or absence of deuterated bonds was easily discriminable and reliable enough to be learnt.

*3: The conditioned isotope response was generalisable between molecules containing the same isotopes. Drosophila trained to avoid deuterium in one molecule avoid it in other molecules.*

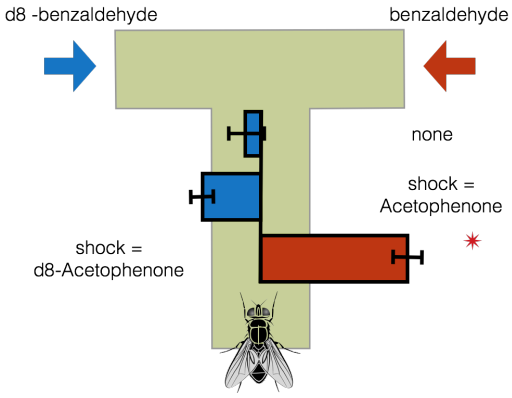



*Figure 7: Drosophila trained to avoid deuterium in one molecule avoid it in other molecules. Redrawn from Figure 3 of Franco et al (Franco, Turin, Mershin & Skoulakis 2011b)*

Flies trained against ACP (deuterated and hydrogenated) and octanol (d- and h-) were exposed to the novel bezaldehyde pair. They demonstrated aversive behaviour exactly as one would expect, those conditioned to avoid deuterium did so and those flies which associated the hydrogenated odorant with foot shocks were counted in significantly greater numbers in the d5-benzaldehyde arm. In only one case was there no significance between the arms and that was for flies trained against ACP. This was explained by the fact that ACP is innately attractive whereas h-benzaldehyde is aversive, and thus the discrimination task was more difficult.

*4: Not only this but they could generalise this quality to other non-deuterated molecules of a similar vibrational spectrum.*

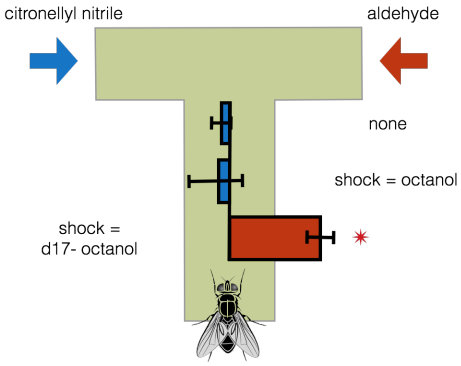



*Figure 8: Drosophila generalise learning to other non-deuterated molecules of a similar vibrational spectrum. Redrawn from Figure 4 of Franco et al (Franco, Turin, Mershin & Skoulakis 2011b)*

The conditioned isotope response was generalisable to molecules without deuterium which nevertheless shared a stretch vibration in the same region as the C-D bond (about 2200 cm-1). Untrained flies did not discriminate citronellal and citronellyl nitrile - compounds which have very similar vibrational spectra although the nitrile has a significant peak around 2150 cm-1 from the nitrile bond. D17 octanol has a similar peak at the same part of the spectrum.

Flies trained to avoid octanol make no distinction between the nitrile and aldehyde odorants but those conditioned to avoid the deuterated octanol avoid the nitrile

significantly. Similarly, those Drosophila trained to avoid the nitrile avoid the deuterated octanol, but those with an aversion to the aldehyde make no such distinction. Interestingly there was no spontaneous aversion to the d8-octanol as shown in the first experiment, it is not mentioned whether the maze was balanced to achieve this.

In essence, the flies were shown to be able to smell the difference between deuterated and non-deuterated odorants and that this discrimination was due to the differing odorant molecular vibrations. It is difficult to see how any other molecular cue could be responsible for the results of this experiment.

Surprisingly the article elicited little in the way of comment or counter-argument from the olfaction community. In a reply published in PNAS (Hettinger 2011) Thomas Hettinger, while accepting that the experiments showed that *Drosophila* could discriminate between deuterated and non-deuterated odorants, maintained that this did not demonstrate that it was through vibration-detection that they did so. His reasoning for this amounted to: humans can't detect isotopes, olfaction isn't a spectral sense, IETS has never been demonstrated in a biological system, and that NADPH cannot generate electrons. He goes on to claim that the large change in the vibrational spectrum with deuteration means that it should be easy to tell the difference between the isotopes and since it isn't that easy, that this cannot be the mechanism. He rightly concludes that "lock and key" vs "vibration" as exclusive mechanisms is a straw man, although this not an argument ever made in the Franco paper.

The authors rebutted in the same issue of the journal (Franco, Turin, Mershin & Skoulakis 2011a), politely pointing out that none of the arguments undermined the findings of the paper.

The paper is an elegant demonstration of the vibrational theory and the findings have been independently replicated twice (Turin, pers comm). It is difficult to find another explanation of the findings consistent with a non-vibrational hypothesis.

The convincing support of the vibrational hypothesis is really the last experiment, the abstraction of the deuteration training to a set of compounds which do not contain deuterium but which do share that vibrational mode. It may be that this is just random chance, that the researchers stumbled across a pair of molecules which caused the same behaviour for another reason. They do not reveal how many other molecular pairs they tried. This is important because if this particular pair was one of thousands then the significance of the finding is much lower. If this is the only such pair, then the predictive power of the hypothesis is extremely high.

Bittner et al (Bittner et al. 2012) published a paper with a combination of a theoretical physical modification of the vibrational hypothesis and a recapitulation of the experiments showing *Drosophila*'s ability to detect deuterated acetophenone.

Their "impulsive scattering" model is a slight modification of Brookes et al's (Brookes et al. 2007) theory which added a spatial component - only vibrational stretches along certain vectors are important in the determination of the odour character.

The authors attempt to confirm the model is accompanied by an experiment in *Drosophila* which does not make any attempt to falsify their new model nor to compare it against the original calculations made by Brookes et al. What they did demonstrate very clearly is the ability of the flies to detect the amount of deuterium within the molecule, they could distinguish between d3-, d5-, and d8-ACP in turn. Taking only the IR spectrum into account, the difference between d5- and d8-acetophenone is negligible and the *Drosophila* ability to tell the difference requires more explanation.

Differences Between Vertebrate and Invertebrate Olfaction

As Hettinger pointed out (Hettinger 2011), insect and vertebrate olfactory systems are very different in a number of ways, and it may be that invertebrate olfactory receptors use a fundamentally different mechanism to detect the odorant. There are several cautions against extrapolating insect olfaction findings to other phyla:

In insect olfaction, odorant binding proteins have been shown to play a large role (Fan et al. 2011). As many as 66 different OBP types are present the olfactory mucus (in *Anopheles gambiae*) (Fan et al. 2011) and most insects have a limited repertoire of ORs (about 60).

The OBPs play a significant role in the ligand recognition step of olfaction, for instance being required for the detection of the classical example pheromones: bombykal and bombykol, of the silkworm moth *Bombyx mori* (Pophof 2004). Some OBPs have been shown to be narrowly tuned (the aphid OBP: SaveONP7 only detects *trans*-β-farnesene, the aphid alarm chemical), whereas others are much broader (Zhong et al. 2012).

A similar role for human OBPs has not been shown, in fact only two, closely related, OBPs have been identified: OBPIIa and OBPIIb (named after their amino acid similarity to the rat OBP2) (Schiefner et al. 2015). Additionally they do not seem to be expressed in the same high concentrations within the nose as in other mammals (Lacazette et al. 2000), and may not be expressed intact at all (Hélène Débat et al. 2007).

Insect olfactory receptors are also different. Unlike in *Drosophila* where genes for development and structure of the auditory system are conserved between mammalian and fly hearing structures (Boekhoff-Falk 2014), there is significant variation in olfaction. For instance, there is little homology with mammalian ORs as measured by a hidden Markov model GPCR detector. (Wistrand et al. 2006). The receptors are expressed as heterodimers

on the cell membrane, paired with an ion channel protein OR83B, now known as ORCO. Activation of the receptor can open the ion channel to begin membrane depolarisation, but the GPCR can still activate a G-Protein as in mammals, giving a longer response. (Wicher et al. 2009). This is in contradistinction to the mammalian OR which is expressed singly in conjunction with the Golf protein. Insect ORs are also "the wrong way round" with the carboxyl terminus of the amino acid chain on the extra-cellular surface (Kaupp 2010), unlike in vertebrates. For a good review of the differences and similarities see Kaupp (Kaupp 2010), the difference are summarised in Table 2.

| *Characteristic* | *Vertebrates* | *Insects* |
|---|---|---|
| *Class* | GPCR | Non-GPCR |
| *Repertoire* | Large, variable | Smaller, constant |
| *Topology* | Heptahelical | Inverse heptahelical |
| *Activation* | Metabotropic | Ionotropic |
| *Pseudogene fraction* | High | None to low |
| *Stoichiometry* | Monomers | Heteromers |
| *One receptor–one neuron rule* | Yes | Yes* |
| *Gene selection* | Stochastic | Deterministic |
| *Expression pattern* | Zonal and random | Zonal and random |
| *Instructive role* | Yes | Unknown |
| *Ectopic expression* | Yes | Unknown |
| *Inhibitory action of odorants* | Rare | Common |
| *Convergence of axons to glomeruli* | Yes | Yes |
| *Glomeruli per receptor type* | Variable, ≤2 up to 20 | ~1 |

*Table 2: Table 2 from Kaupp (Kaupp 2010) differences and similarities between insect and vertebrate GPCRs*

So it is with some care that the findings of Franco et al (Franco, Turin, Mershin & Skoulakis 2011b) and others (Gronenberg et al. 2014) (Bittner et al. 2012) should be extrapolated to human olfaction, but since the structure of the ORs are still that of 7TM receptor proteins, the ability of *Drosophila* to detect molecular vibrations suggests that this mechanism may be present in in vertebrates too.

**Deuteration Experiments in Vertebrates**

The only non-human vertebrate deuteration-detection experiments were performed by Hara (Hara 1977), demonstrating that the whitefish *Coregonus clupeaformis* were able to detect glycine and demonstrated an innate aversion to the deuterated form. Fish olfaction is different to that of terrestrial animals in that they can detect amino acids dissolved in water although the sense is still mediated by the olfactory bulb (Caprio 1975). Again, all this demonstrates is that the fish can detect deuterated compounds, not necessarily that a vibrational mechanism is used, nor that it is a receptor-level function.

Turin (Turin 1996) suggested that the protons within glycine were all potentially exchangeable with those in the surrounding water, invalidating the experiment. Wade (Wade 1999) pointed out that the methylene hydrogens or deuteriums cannot exchange with the protons in water at physiological pH.

**Human Experiments**

In some ways Turin's 1996 paper was a truly psychophysical theory, he attempted to elicit the mechanism of olfaction using mainly perceptual clues, a difficult task and, given the complexity of the system, one with many traps for the unwary.

Several attempts have been made to investigate the vibrational hypothesis of human olfaction by psychophysical methods. Following the publication of Turin's paper in 1996, the first published paper was Haffenden et al (Haffenden et al. 2001) examining benzaldehyde

deuteration in a duo-trio test. Their version of the test was later called "anomalous" in the Keller- Vosshall paper of 2004 and was criticised for not being double-blinded (the method does not specify the blinding of the experiments although each sample was labelled with a code presumably known to the experimenters). Haffenden used 30 trained panellists and exposed each to three different trios, once each, with no training. The comparisons were between benzaldehyde and one of: $^{13}$CHO-benzaldehyde, $^{13}C_6$ -(ring)-benzaldehyde or benzaldehyde-$d_6$. No purities were specified. The panellists were allowed only one sniff of the samples and one minute between sets for recovery. The results were most convincing for the deuterated-nondeuterated comparison with 23 out of 30 able to correctly identify the "odd one out".

It was the paper by Keller and Vosshall in 2004 (Keller & Vosshall 2004) which was widely perceived as the final nail in the coffin of the vibrational theory. Accompanied by an extremely aggressive editorial (Anon 2004), the paper examined three key claims made by Turin in the 1996 paper: that a mixture of guaiacol and benzaldehyde would smell like vanilla, that aldehydes of an odd number of carbon atoms smelled similar to each other in a way that those with an even number did not, and that acetophenone (AP) and perdueterated acetophenone should have distinguishable smells. The tests were double blind, using naive subjects and with standard psychophysical tests. There were no results supporting the thesis that "molecular vibrations alone can…explain the perceived smell of an odorous molecule".

To examine the "vanilla-ness" of benzadlehyde/guaiacol mixture they used a 13 point score (1 = no vanilla, 13 - extremely vanilla). The subjects were asked to rate the vanilla character of the seven possible combinations of components. There was wide overlap in the rating of all samples, not one was significantly more "vanilla" than the others - not "smells

like vanilla" but "has a vanilla note". Although this was reported as a negative finding, it was strictly a finding of "no result", at 90% CI the "vanilla-ness" of benzaldehyde alone could have fallen between 0 and 8, with Vanillin falling between 4 and 13. Relying on untrained human smellers to detect and identify a "note", seems a very weak method of assessing this.

For the aldehyde carbon chain length comparison, subjects were asked to rate the difference between randomised pairs of aldehyde samples differing by up to 6 carbons. Pairs of odd or even numbered chain-length aldehydes were no more likely to be perceived as different than those which featured one of each.

Finally, and most importantly for this project, Keller and Vosshall examined the perception of difference between perdeuterated (d8-AP) and hydrogenated acetophenone. Initially the subjects were asked to rate the similarity of a presented pair of AP/AP, AP/d8AP or d8AP/d8AP on a 10 point scale. There was no significant perception of difference. Additionally, a triangle test to distinguish between the two was performed with the well-known carvone enantiomer pair used as a control, R- and S-carvone were easily discriminated, but the ability to determine the difference between acetophenone and d8-AP was not significant across a range of dilutions. Finally, a duo-trio test was used in a subgroup who had been successful in identifying AP vs d8-AP in the previous session. This subgroup did no better that chance.

The drawbacks of the experiments as a whole were the lack of pure samples (purity ranged between 99.9% and 99.3%); the use of untrained, naive smellers, without repeat trials; and a lack of control for nasal disease. No information as to the timing between samples and sniffs was provided.

The relatively (for olfactory studies) low purity of the odorants could have given a number of conscious or unconscious clues to the participants. Since some molecules (such

as p-vinylguiacol (Baek & Cadwallader 1999)) can be detected in concentrations as low as 10 parts per billion, it is vital that all samples in olfactory psychophysical testing are as pure as possible. (Hence the use of GC pure samples in this project).

Similarly, naïve smellers who might have no olfactory ability at all (this was not demonstrated to be present) and may not be the best subjects to elicit a fine odour quality distinction.

With all these caveats, the paper did not show any support for the vibrational hypothesis, but cannot be considered to be a definitive contradiction of the theory. Perhaps theoretical physics can guide further research and provide better experimental direction.

**In Vitro Experiments**

Published after most of the experimental work in this thesis, "The Implausibility of the Vibrational Theory of Olfaction" (Block, Jang, Matsunami, Sekharan, et al. 2015) by Block et al in 2015, was published partially as a response to some of the published work of this thesis. I report on the basic findings here as part of the overall literature review and consider the relationship between the findings of this thesis and the findings of Block et al in the discussion.

This paper considered the theoretical work on the vibrational hypothesis and published the results of a screening of 330 different human ORs in a large scale heterologous expression system against a series of ligands and their isoptomers, as well as investigating the variation of activation on ten different ORs and their ligands.

Block et al synthesised seven isoptomers: d30-muscone, d4-, d24-, and d28-cyclopentadecanone and MTMT (8), MTMT- d5, as well as bis(methylthiomethyl) disulfide. The muscone isoptomers (deuterated and non-) were introduced to the 330 human

receptors the Matsunami method has been able to express in a previously described high-throughput heterologous HANA3/luciferase at an unknown exposure concentration.

Only a few receptors were activated by the muscone isoptomers, OR5AN1 having the largest response.

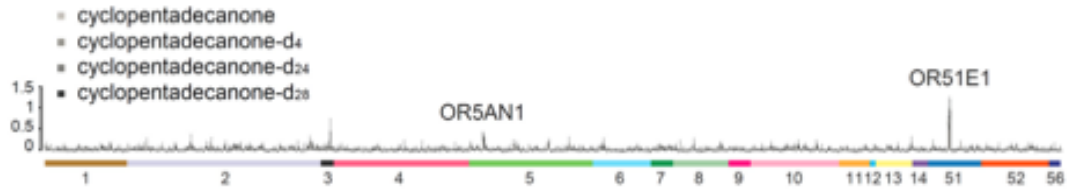



*Figure 9:Block et al (Block, Jang, Matsunami, Sekharan, et al. 2015) Figure S3.1: screening of human ORs for all four isoptomers of cyclopentadecanone*

The two odorants demonstrated were then examined in more detail. Only the previously reported (Shirasu et al. 2014) OR5AN1 showed significant activity against the odorants and did not show any altered response to any of the deuterated musks. The researchers also exposed the receptor to deuterated and non-deuterated cyclopentadecanol (to which it is weakly positive) and cyclopentadecane, to which it does not respond at all, there was no variation in signal between the deuterated and non-deuterated versions of these molecules either. Another two ORs: OR5M9 and OR51E1, which were strongly but not significantly activated, were later published (Block, Jang, Matsunami, Batista, et al. 2015) and also did not demonstrate any activity to the musk odorants.

Block et al went on to examine the variation in the response to isoptomers of benzaldehyde and acetophenone, using the mouse ORs known to respond to these odorants. There was no significant variation response to either.

Importantly, for these receptors, another set of isoptomers was also checked, the $_{13}$C versions of the odorants: acetophenone-α,β-$_{13}$C2, acetophenone-$_{13}$C8 and $_{13}$CHO-bezaldehyde-$_{13}$C1. As discussed, Klika (Klika 2013), had previously suggested that these were a vital negative test of the vibrational hypothesis, in that these isotopes do not significantly alter the vibrational frequencies but they do alter the association/dissociation and on/off rates. The carbon isotope analogues fared no differently to their $_{12}$Carbon counterparts in activating their various receptors. As Klika says: "For the vibrational theory to endure, it must at least be able to stand this test; though unfortunately if vibrational theory does indeed pass the test, it is not substantiated by it." Although not demonstrating the vibrational alteration of receptor-ligand interaction this does exclude other mechanisms in the odorant-receptor interaction of the isoptomers other than vibration.

They further examined fourteen other receptor/odorant pairs, including MTMT and related odorants, ligands for the mouse receptor MOR244-3 which some of the authors had previously demonstrated require copper for ligand binding (Duan et al. 2012). Again, none demonstrated variation in luciferase activity compared to the non-isoptomer compounds.

In all of the odorant-ligand pairs, not a single variation in the luciferase activation was demonstrated between the isoptomer pairs. For the 13C isoptomers this was a support (or at least non-contradiction), but for the deuterated molecules, this would not have been predicted from the models and is a significant problem for the vibrational hypothesis.

The paper also included a detailed analysis of the current theoretical work as discussed below.

**Theoretical Analysis of the Vibrational Hypothesis**

For over a decade no-one examined the physics claims of the vibrational hypothesis until Brookes et al (Brookes et al. 2007) proposed a model of the mechanism which used

a semi-classical model with only the odorant oscillator as the vibrational mode which acts to facilitate the electron transfer. Using some reasonable assumptions, they derived the Huang-Rhys factor for the system, a measure of the vibrational transition probabilities in an electronic transition. This was low but still plausible given the assumptions that the receptor is finely tuned to the vibrational quantum of the odorant (a feature explicit in the vibrational hypothesis) and the reorganisation energy of the receptor is low.

Block et al (Block, Jang, Matsunami, Sekharan, et al. 2015) criticise these assumptions for the lack of experimental support, especially in that the reorganisation energy used in the equation is an order of magnitude smaller than modern estimates, although this can be modified by altering other assumptions about the resonance conditions. Solov'yov (Solov'yov et al. 2012) reviewed the conditions required for a biologically plausible inelastic tunnelling mechanism in the olfactory receptor. They also comment on the requirement for a small reorganisation energy, below 0.1 eV, a value which is substantially lower than current estimates for other receptors (Block, Jang, Matsunami, Sekharan, et al. 2015). More specifically they examined the variation expected in deuterated compounds and concluded that the experimental plan of this project was a good test of the theory:

*"For an experimental verification of the vibrationally assisted mechanism of olfaction one should carry out studies of odorants with identical (or similar) surface properties inducing different responses in the olfactory system."*

They go on to confirm that deuterated molecules fulfil this role acceptably.

The role of vibration in *Drosophila* olfaction specifically was investigated by Eric Bittner and team that same year (Bittner et al. 2012). The experimental work is discussed in the insect olfaction section *supra*. The theoretical addition to the hypothesis was the

addition of a spacial element to the model, although Turin referred to the unfavourable position of an electron scattering group as one of his 'ways of being odourless' in the original paper (Turin 1996). Brookes and Horsfield also included a spatial element in their updated 'swipe card' model (Horsfield 2012) that same year. Following the publication of some of the work in this thesis (Gane et al. 2013) there was some interest in modelling the requirements for the 'muskiness' odour. Maia et al (Maia et al. 2014) used a computational method of frequency and intensity estimations to compare the vibrational bands for similar musk and non-musk odorants. They found that three of the four bands Turin predicted as musk frequency bands were able to distinguish between the two groups, but frequencies in Turin's "carbonyl stretching mode" range around 2000 $cm^{-1}$ were not exhibited by any of the molecules. Carbonyl groups gave frequency peaks around 1680–1800 $cm^{-1}$ and were common to musk and non-musk molecules and so this band was rejected as being a useful determinant of musk odour.

These theoretical approaches were aimed at modelling the receptor activation step. A different approach was to look at the explanatory power of the theory itself. Gabler et al (Gabler et al. 2013) used a machine learning approach to evaluate this, expressed in a set of descriptors derived from the vibrational spectra of odorants. Using the largest open database of *Drosophila* odorant-ligand interactions - DoOR (Galizia et al. 2010) they trained machine learning algorithms using "classical" physiochemical descriptors, a previously suggested (Haddad & Sobel 2008) optimised subset of these and vibrational characteristics. There was a high degree of similarity between the three. The best performing algorithm was trained on the entire 1460 descriptor set, whereas the lower dimensional vibrational and optimised sets were only slightly poorer at predicting the activity of the ligand-OR pair. The vibrational descriptor consists of twenty-seven dimensions: the pattern of wavelengths across the spectrum, and one free parameter: $\sigma$,

the kernel bandwidth. To use only a twenty-seven-dimensional descriptor is obviously more efficient than having to analyse just under one-and-a-half thousand dimensions, especially if their performance is essentially identical.

They were quick to point out that this was not to be construed as a proof of the vibrational hypothesis but rather that a molecule's vibrational spectrum is a result of its structure and is essentially a well-condensed version of the same information as the physiochemical descriptor set. This may be the explanation for the predictive power of the vibrational hypothesis even if it is wrong: vibration is a good summary of the characteristics that do predict the odour. Whether these arise from a direct evaluation of the vibrational modes within a molecule requires an objective demonstration of the effect.

**Summary of Current Literature On The Vibrational Hypothesis**

The literature around the vibrational hypothesis was contradictory and of mixed quality, especially at the start of the project at the turn of the last decade. The hypothesis claims that the central recognition step in the activation of the olfactory receptor is a phonon-assisted electron tunnelling which relies on certain vibrational modes of the bonds within the molecules. To demonstrate role of molecular vibration detection in human olfaction requires showing a change of odour quality perceived by humans in molecules in which the vibrational, but not shape, characteristics have been altered. Even if this alteration of odour quality was demonstrated, further work would be required to show whether this was a receptor-level phenomenon.

## The Human Olfactory System

I discuss here the overall structure of the olfactory sensory system with special attention to the processes which can affect the perception of odorant molecules, before undertaking a slightly more in-depth look at the olfactory receptor and its roles. The olfactory receptor is a G protein–coupled receptor (GCPR) and I examine the current

knowledge of the activation processes of the class of GPCRs as a whole in the subsequent section, finally an overview of the methods available and methods used in this project to investigate the human sense of smell.

## The Sense of Olfaction

The sense of olfaction is the detection and identification of inhaled volatile molecules and is, when combined with gustation, as "chemoreception", the oldest sense. Famously in humans it has become subordinated to the newcomers: vision and hearing, but it nevertheless plays an important role in our perception and enjoyment of the world. The anatomy and physiology of smell have many features which reflect the sense's evolutionary antiquity.

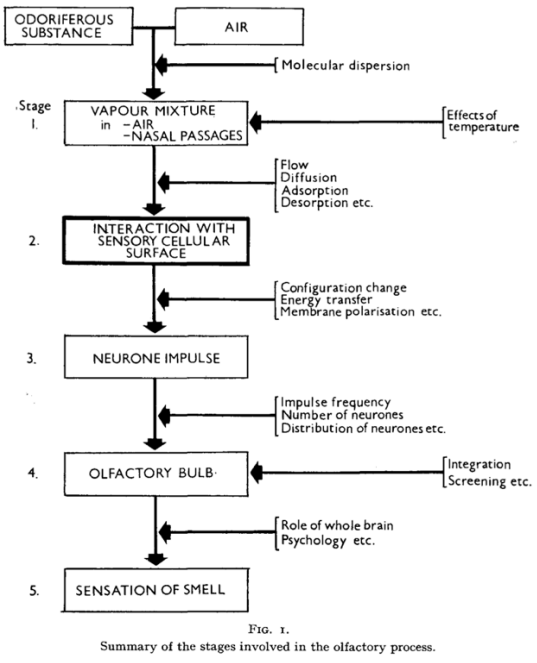



FIG. 1.
Summary of the stages involved in the olfactory process.

*Figure 10 The Stages of Olfaction from Douek (Douek 1967)*

In terms of the gross stages of olfaction, little has changed since Douek (Douek 1967) published Figure 10 in the JLO almost fifty years ago. Much more is known about the individual interactions at each stage: "interaction with the sensory cellular surface" for

instance is now known to be a receptor interaction, but in general terms, his outline is correct.

As the molecule enters the nose it must traverse the cavity to the olfactory area in the olfactory niche high on the roof of the nose. The odorant is small, volatile, and hydrophobic, allowing it to dissolve into the overlying olfactory mucus. Here it may bind with a class of general-purpose binding molecules known as Odorant Binding Proteins (OBP) or react with metabolically active constituents of the olfactory mucus such as enzymes. The odorant, odorant-OBP complex or metabolites must then move through the mucus layer to the cilia of the Olfactory sensory neuron (OSN) where they may activate olfactory receptors (ORs) on the OSN's cell surface. Activating an olfactory receptor causes its linked G-Protein ($G_{olf}$) which, when activated, causes an alpha subunit dissociation and an increase in intracellular cAMP. This activates the transmembrane cAMP-activated cationic channel: CNG, with depolarisation of the neuron as the end result. The depolarisation signal passes along axons through the cribriform plate to one of two glomeruli specific to that receptor in the olfactory bulb (OB). There is some further processing before second and third order neurons pass back along the olfactory tract to their relative lateral (primary), intermediate and medial (secondary) olfactory areas of the rhinencephalon, forming the representation of the odour percept within the brain.

## Olfactory Mucosa

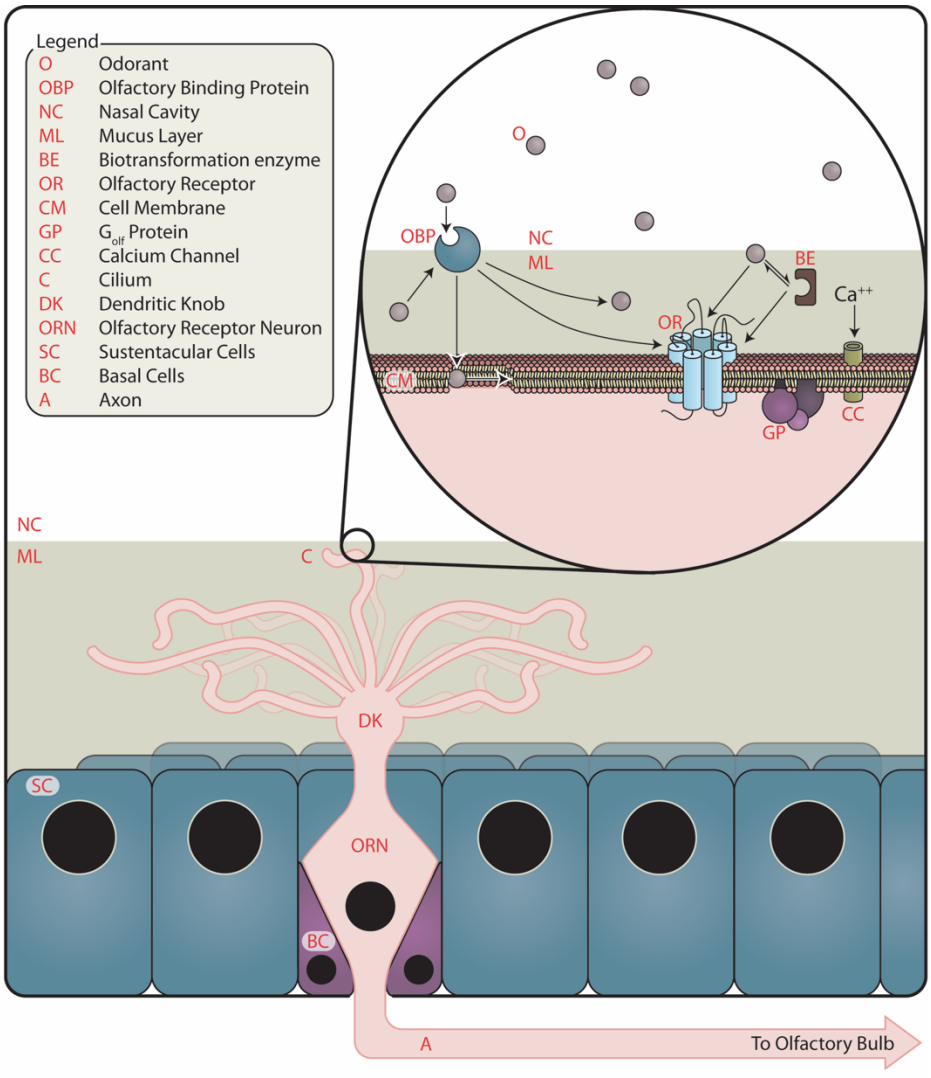



*Figure 11 : The olfactory sensory neuron and perireceptor events.*

## Olfactory Mucus and its Contents

The olfactory system proper can be said to begin with the mucus layer overlying the olfactory epithelium of the olfactory cleft and superior turbinate. About 10-40µm thick in humans (Graziadei 1971) and produced by Bowman's glands on the lamina propria of the olfactory mucosa (Hadley et al. 2004) and elsewhere (Steinbrecht 1998), it contains the secreted Odorant Binding Proteins (OBP) and many other proteins, including a wide range of oxidative and hydrolytic enzymes.

The mucus can affect the olfactory percept in three ways; its liquid nature acting as a separation column, the odorant binding proteins (OBPs), and the enzymes within.

The liquid nature of the mucus means it functions as a separation column to fractionate the odorant particles (Mozell 1970) borne out by the fact that solubility of odorants correlates with pungency (Cometto-Muniz et al. 1998) and olfactory threshold (Cometto-Muñiz & Abraham 2010a) (Cometto-Muñiz et al. 2008)

Generally, the threshold for a series of odorants decreases with increasing hydrophobicity. This has been interpreted as an effect of the mucus, but it could also be a result of hydrophobic bonding with the receptor. *In silico* modelling has shown that the largest energetic barrier to binding is at the entrance to the binding pocket and that subsequent entry required deformation of the receptor (Dror et al. 2011). This entry barrier might be the result of the loss of hydration sheaths from around the ligand and receptor. This might explain why, the more hydrophobic an odorant, the lower its threshold; as this entry barrier step might be lower or even result in an energy gain as the water molecules are removed.

Within the olfactory mucus there are as-yet ill-understood processes acting on the odorant molecules before they reach the cell membrane and the olfactory receptor: interaction with the odorant binding proteins, biotransformation enzymes and other chemical processes.

**Odorant Binding Proteins**

Odorant Binding Proteins (OBPs) and their related Pheromone Binding Proteins are secreted proteins of the lipocalin family and are able to bind numerous odorants of diverse chemical structures, with a higher affinity for aldehydes and large fatty acids (Briand et al.

2002). They have a molecular weight of about 20 kDa (Steinbrecht 1998),and may function best as dimers. (Ikematsu et al. 2005) (Nespoulous et al. 2004)

Debat and colleagues (Hélène Débat et al. 2007) used several techniques to identify 83 different proteins within the mucus, but the human odorant binding protein was detected in rather low concentrations compared to other mammals such as cow, pig, rabbit and others. No total hOBP was detected by the Western blot assay and several degradation fragments were found in high concentration, leading the authors to conclude that the protein was subject to a high degree of degradation within the mucus and wondered whether, given this, the OBP could play an important role in human olfaction.

Never the less, like the olfactory mucus layer the OBP is conserved amongst terrestrial animals and likewise the precise role of OBPs is still much discussed. There is evidence for functions including: solubiliser (Briand et al. 2000), biocarrier (Yabuki et al. 2011), cofactor (Vidic et al. 2008), deactivator (Steinbrecht 1998) or filter: "pre-selecting" the compounds which interact with the receptor (Breer 2003).

Importantly, OBPs can alter the binding and affinity of odorants for their receptors (Vidic et al. 2008) and any model system without OBPs may only have a partial representation of the olfactory sense.

**Enzymes**

It is unlikely that odorant binding proteins act as enzymes but the olfactory mucosa has long been known to be a highly metabolically active tissue. One of the confounders of in vivo experimentation is the potential for multiple unknown chemical changes to the odorant before it reaches the receptor.

Since the early '80s interest has been directed at Cytochrome P-450 in the olfactory epithelium, where there is, weight-for-weight, as much C-P450 as in the liver (Dahl et al. 1982) . Cytochrome-P450olf1 is a specific subtype in rats and cows (and probably in humans). (Nef et al. 1989) It is part of a cytochrome subfamily whose "members are known to catalyse the conversion of numerous hydrophobic compounds" including many odorants. Since then the genomic revolution has allowed several "nasal mucosa-predominant" enzymes to be found (Xiuling Zhang et al. 2005), which have been shown to have expression levels commensurate with those in the liver.

The effect of these cytochromes on odour was not fully appreciated until another cytochrome: CYP2A13, was shown by workers at Givaudan (Schilling et al. 2009) to have a significant impact on odour character. During a routine screening of the activity of CYP2A13 it was noticed that the breakdown product of the nor-terpenoid, an allylic alcohol 4,8-Dimethyl-5-(1-methyleneethenyl)bicyclo[3.3.1]non-7-en-2-one, had a strong raspberry scent whereas the original molecule: (5,9-Dimethyl-6-(1-methyleneethenyl)bicyclo[4.3.1]dec-8-en-3-one), was said by some to have a woody character and others a raspberry odour.

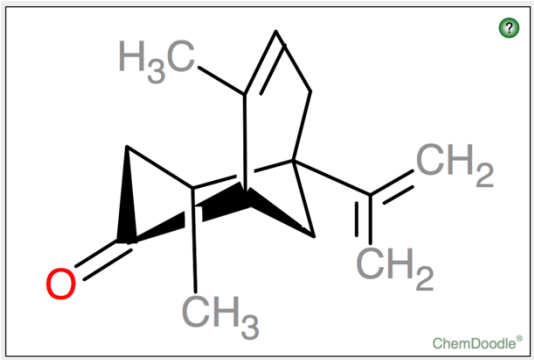



*Figure 12: 5,9-Dimethyl-6-(1-methyleneethenyl)bicyclo[4.3.1]dec-8-en-3-one. Elicits a woody odour if not metabolised to the allylic alcohol in Figure 2.6. Structure drawn in ChemDoodle (iChemlabs)*

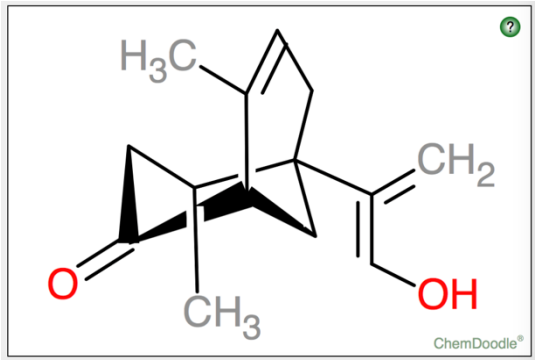



*Figure 13: 4,8-Dimethyl-5-(1-methyleneethenyl)bicyclo[3.3.1]non-7-en-2-one. Structure drawn in ChemDoodle (iChemlabs)*

An odour panel smelled the molecule in a mixture with the otherwise fairly odourless CYP2A13 inhibitor (E)-3-(cyclopropylmethylene)octan-2-one. Of those who had detected a raspberry note in the original, two thirds reported the odour as no longer smelling of raspberry. Inhibiting the function of the cytochrome removed the smell of raspberry. This odour was not a function of the receptor detecting a molecule but rather the metabolism of the molecule within the olfactory mucus to breakdown product which presumably stimulated a different set of receptors. The details of these experiments are obfuscated in several patent filings from Givaudan (Schilling & Schilling 2009), but the reported results (Sell 2014), although not peer-reviewed, are that the phenotype for perceiving the raspberry note from the nor-terpenoid co-segregates with isoforms in the genes coding for the enzyme CYP2A13. The genetic cause for this specific anosmia is not a receptor, but an enzyme variation. This is the reason for including a range of non-receptor genes in the Galaxolide STV experiment (chapter 5), and why an exome-wide approach might be valuable too.

Nagashima and Touhara (Nagashima & Touhara 2010) demonstrated this same effect in mice.

It seems likely that these enzymes exist in a complex network of interconversion between receptor agonist/partial agonist/antagonist/enzyme catalyst/inhibitor which may further allow discrimination between sterically similar odorants, similar to a mechanism proposed in 1950 by Kistiakowsky (Kistiakowsky 1950), discussed below.

**Non-Enzymatic Changes**

Even without enzymatic changes the olfactant molecule can undergo chemical changes such as hydrogenation from a deuterated state, which is a caution to the use of the deuterated molecules as psychophysical probes. Some authors have suggested that there may be chemical reactions between the odorants and OBPs and perhaps even ORs. For instance Li et al (Y. Li et al. 2014) showed that, of the ORs reacting to aldehydes, some were likely to be activated instead by the *gem*-diol form (two -OH replace the two terminal -H) spontaneously or perhaps catalytically formed in the olfactory mucus.

**Summary: A Pre-Receptor Metabolic Network**

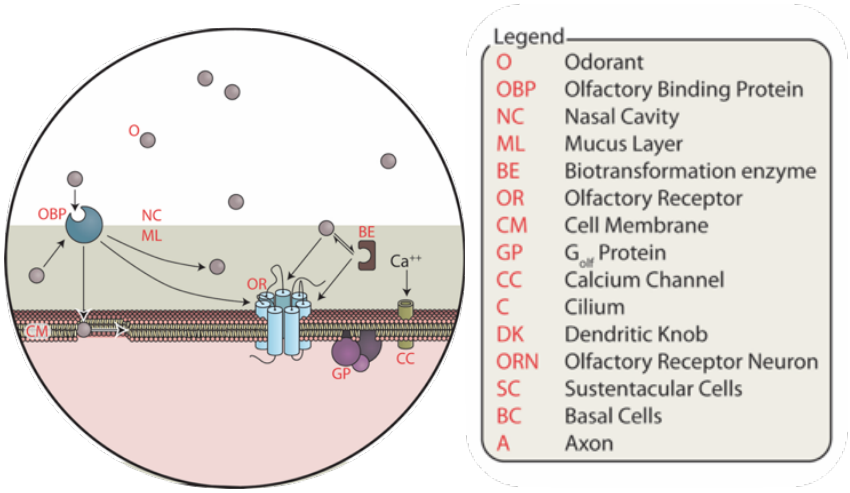



*Figure 14: A metabolic network of odorant interaction between odorants, odorant binding proteins, enzymes, and olfactory receptors*

The above evidence supports Kistiakowsky's (Kistiakowsky 1950) suggestion in the 1950s that there is a network of complex interactions, reactions and metabolism in the nasal mucus and olfactory mucosa. Any molecule introduced into the nose must traverse a a

network of solution partitioning, trafficking by binding proteins, enzymatic and non-enzymatic metabolism, partial and inverse agonism, allosteric and other interactions; all of which may change its interaction with the olfactory receptor.

The search for a simple structure-odour relation, such as the vibrational hypothesis is therefore less likely and any psychophysical demonstration of altered odour must be accompanied by receptor level evidence.

**Olfactory Epithelium**

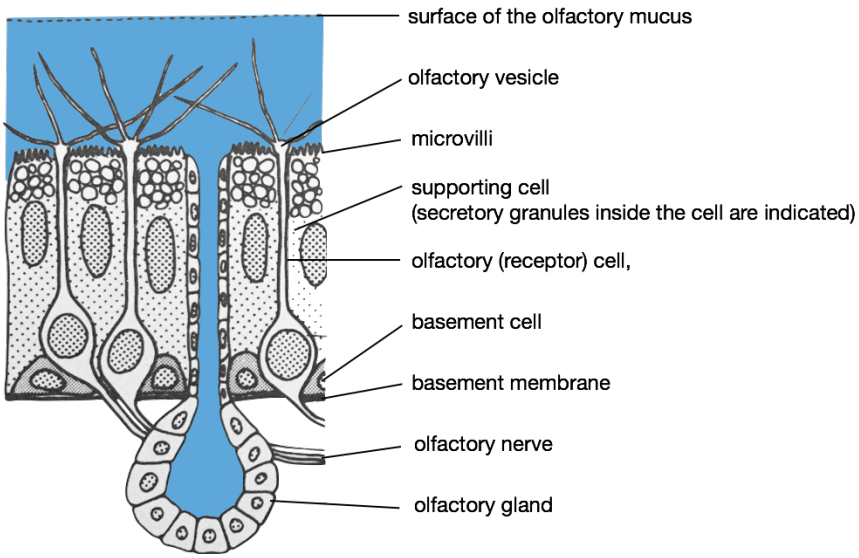



*Figure 15: A schematic diagram of olfactory epithelium, colorised and relabelled Figure 1 from Takagi "biophysics of smell" 1978 (Takagi1978):*

The olfactory epithelium is the one place where the central nervous system is directly exposed to the external environment, albeit behind a layer of mucus and some torturous airways. This is a specialised sensory epithelium containing the mature and immature Olfactory Sensory Neurons (OSNs), some mucus-producing goblet cells and supporting (sustentacular) cells. It is attached to bone via a lamina propria that is characteristically thick

and contains the Bowman's glands previously mentioned and the axonal processes of the neurons. (Jafek et al. 2002)

**Olfactory Receptor Neuron**

Each OSN is a bipolar neural cell with multiple (10-50) fine cilia (about 0.3 microns in diameter) projecting off a single dendritic knob within the mucus layer. These cilia are covered in one of about 350- 400 types of olfactory receptors (Malnic et al. 1999) per neuron and it is on their surfaces that the odorant molecules are detected. The body and nucleus of the cell lie within the olfactory mucosa layer and the axons of the OSNs project upwards through the cribriform plate to the olfactory bulb. The axons are wrapped in a special form of Schwann cell, the olfactory ensheathing glia, which is thought to allow the constant regeneration of the neurons (Kafitz & Greer 1999) from the basal stem cells as the mature cells die off; one of only 2 places this has been demonstrated in the adult human CNS (Moreno-Flores et al. 2002).

OSNs are not merely the vehicles for receptor expression, there is active modulation of their activity by the surrounding environment, hormonal and neuronal(Hall 2011), such as muscarinic receptors(Ogura et al. 2011), and noradrenaline (Arechiga & Alcocer-Cuaron 1969) in humans.

One of the fundamentals of the olfactory system is neuronal identity: each OSN selects and expresses only one type of olfactory receptor, the "one neuron-one receptor rule".(Serizawa et al. 2004) The exact mechanism behind receptor choice and the enforcement of this "singular expression" remain poorly understood (Vassalli et al. 2002). Since the genes express copy-number variation too,( Endicott et al. 2008) to preserve the odour mapping it is imperative that there is monogenic and monoallelic (Chess et al. 1994) expression of the chosen gene.

The gene selection process is still ill-understood but seems to be a combination of regulatory elements (Serizawa et al. 2003) (Khan et al. 2011) (Markenscoff-Papadimitriou et al. 2014), including the RTP protein (Sharma et al. 2017) as well as epigenetic phenomena (Magklara et al. 2011).

The maintenance of this choice is via a feedback loop, which requires an active OR. Pseudogenes can be expressed but if the protein is not functional, the choice is "switched" to another active allele (Shykind et al. 2004) (Feinstein et al. 2004), probably mediated via cAMP (Imai et al. 2006), the unfolded protein response (UPR) and alteration of adenylyl cyclase transcription (Dalton et al. 2013), as well as feedback from activation of the G-protein via the βɣ subunit (Ferreira et al. 2014), supressing OR expression via histone methylation.

The one neuron - one receptor rule has been questioned (Mombaerts 2004) but current evidence is fairly robust. The OSN can be identified by only one receptor, it is "wired" to the glomerulus for that receptor and the glomerulus is therefore the representation of the molecular detecting "receptive field" of that receptor.

## The Olfactory Receptor

The olfactory receptors were first discovered by Linda Buck and Richard Axel in the early 1990s (Buck & Axel 1991). They provide the essential discriminatory step in the perception of odour each receptor being "tuned" to a set of odorants, some more broadly so than others. (Firestein 2001)

I review here the state of knowledge on the structure of the olfactory receptor and its genetics, what is known about GCPRs in general and their activation processes, which must constitute the alternative model for the activation of ORs.

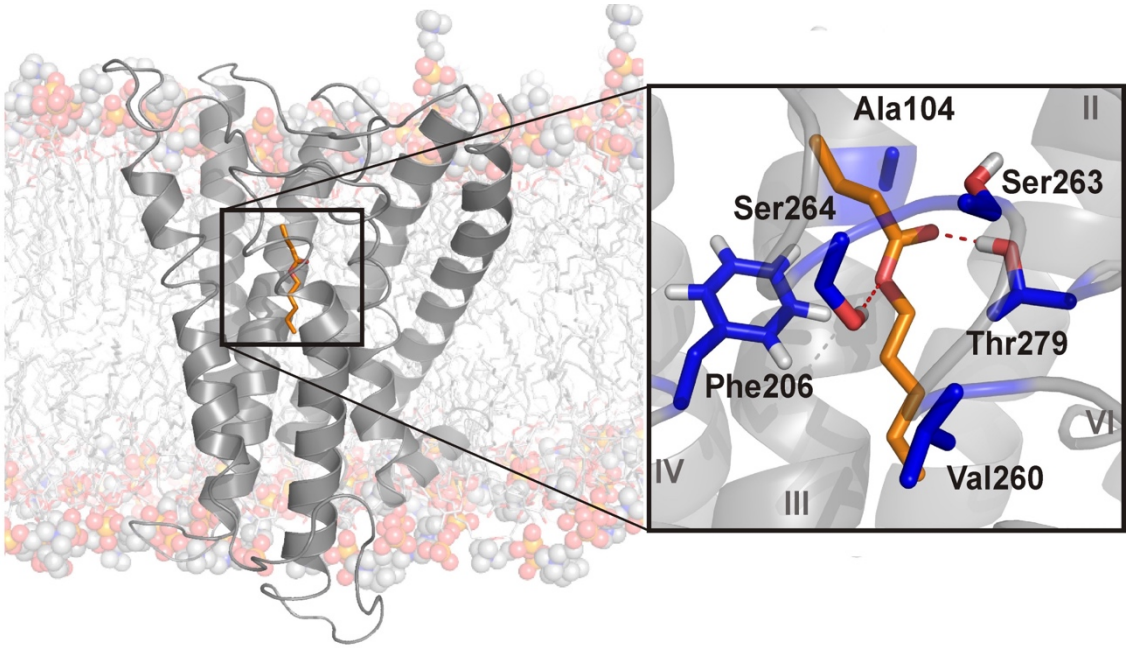



*Figure 16: Interaction of receptor and fragrance: Olfactory receptors (grey) detect odorants (orange-red) via certain amino acids in the binding site (blue/white/red). Hydrogen bonds (dashed lines) are decisive here. © Lian Gelis und Steffen Wolf. http://aktuell.ruhr-uni-bochum.de/mam/images/pi2011/gelis-angewandte.jpg accessed 2017-08-01*

Olfactory receptors are known to have seven transmembrane domains with an extracellular N-terminus and the C-terminus intracellularly. (Kolakowski 1994) They have not been crystallised yet and much about their structure remains unknown. Homology mapping (deriving the structure based on similarity to the sequence of other known proteins) suggests the seven transmembrane domain shape but the true structure remains elusive and because of this there is still much speculation about the exact properties of odorants which the receptors detect (Zarzo 2007).

The genes for ORs comprise approximately 1% of the genome, about 1500 genes in mice and 900 in humans. Humans (Niimura & Nei 2003) are remarkable in that so many of our genes have become non-functional by the inclusion of errors such as premature stop codons. These errors are much higher than in the mouse or even other primates. (Gilad et al. 2003) Approximately one thousand genes are estimated to be expressed in rodents

(Xinmin Zhang & Firestein 2002), humans are thought to manage less than three (Young, Friedman, Williams, Ross, Tonnes-Priddy & Trask 2002a) to four (Mainland et al 2014a) hundred.

Olfactory receptor genes are highly conserved, and are usually coded from one exon (Reed 2004).They are mostly found in clusters but some occur singly and they are found on all human chromosomes apart from 20 and Y. (Glusman et al. 2001) Approximately forty-two percent of the genes are found on chromosome 11 which is also the only one which contains genes for Class I receptors which were, until recently, assumed to be unique to fish and amphibians.

The evolutionary genetics of the human olfactory receptors are fascinating. A review of the genomes shows that, along with cetaceans (Kishida et al. 2007), the higher primates have lost about half of their expanded mammalian inheritance of ORs (Niimura & Nei 2007) which has almost doubled since mammals diverged from the monotremes. It is obvious why marine mammals would depend less on olfaction for airborne volatiles but less obvious why this is so for higher primates.

The loss of functional receptors is not uniform amongst individuals within a species. Menashe et al (Menashe et al. 2003)in 2003 undertook a genomic analysis of 189 individuals showing an amazing 178 different patterns of inactivation amongst the receptors. That is to say: 178 different "suites" of active receptors, where one may have receptors {ABCDEF... etc.} active, another suite might have {*B*DEFG...} where A and C were inactive. If the perception of an odorant is dependent on the relative strength of activation of a range of receptors (its receptor profile) this means that almost no two noses are the same! Although the noses will correctly identify an odorant as the same, the "qualia", experience of "what it is like" to the smeller will differ (Nagel 1974).

This inter-individual variation in receptor genomes leads to a phenomenon known as a specific anosmia, while the general olfactory ability is preserved, the subject cannot smell a specific odour. The role of specific anosmias in identifying ORs is discussed below.

**Current Understanding of G-Protein Linked Receptor Function**

Because of the lack of crystal structures for Olfactory receptors (ORs), knowledge of the activation processes, and therefore the role of quantum vibrational detecting effects is lacking. But there is more known about the activation of e G-protein linked receptors in general. This section reviews the state of the field in GPCR research in general and what is known about the activation of these molecules.

G protein linked receptors (GCPRs) have been estimated to be responsible for the activity of over 30% (Beck Sickinger & Budisa 2012) to 60% (Sell 2014) of drugs, including propranolol, antihistamines, dopamine etc. As such they are intense focus of pharmacological research, and the Nobel Prize in 2012 was given to Lefkowitz and Kobilka for their advances in the understanding of the function of these receptors (Anon 2012).

All GPCRs are members of the seven transmembrane helix receptor family (Rosenbaum et al. 2009) and are usually classified by sequence. There are several classification systems (Schiöth & Fredriksson 2005) but in all, olfactory receptors are classified as rhodopsin-like.

In the last six years, over the course of this project, there has been an explosion in the understanding of G protein linked receptor structure and activation (E. Ghosh et al. 2015). At present, there are over 47 crystal structures published of thirteen receptors, unbound and bound to both agonist and antagonist molecules, all of them class A receptors.

Before the technical breakthroughs in crystallisation at the end of the last decade, other techniques and modelling had made some predictions for the activation steps of

GPCRs. Techniques such as site-directed spin labelling demonstrated that the sixth transmembrane helix (TM6) underwent a rigid body movement away from the body of the receptor on activation (Altenbach et al. 2008). It was proposed that the highly conserved "DRY" motif (Aspartate-Arginine-Tyrosine) at the intracellular end of the third transmembrane helix (TM3) containing an arginine residue which forming an 'ionic lock' with a glutamate on TM6 (Ballesteros et al. 2001) destabilising the inactive state of the receptor, and numerous site-directed mutagenesis studies (Rasmussen et al. 1999) showed that it was required for activation in some receptors, but not all (Chung et al. 2002) such as the α2A adrenergic receptor. Spin-labelling once again showed that disruption of this ionic lock resulted in the TM6 movement which allowed the hetero-trimeric G Protein to bind within the gap created, at least in Rhodopsin (Altenbach et al. 2008) and the β2 adrenergic receptor (Yao et al. 2006) (Gregorio et al. 2017)

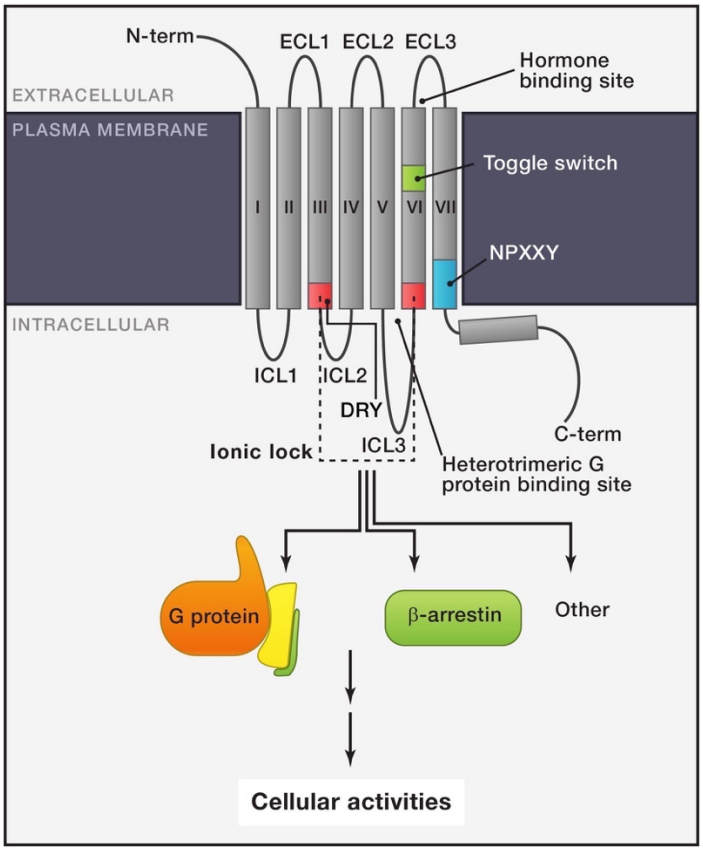



*Figure 17: Figure 1. from Audet and Bouvier (Audet & Bouvier 2012) GPCR topological organisation and signalling paradigm.*

The structure of Rhodopsin was first solved to an acceptable resolution in 2000 (Palczewski et al. 2000) but it was not until seven years later that other GPCRs, bound to diffusible ligands were published (Cherezov et al. 2007) (Rasmussen et al. 2007), the result of many technical breakthroughs. Now, high-resolution structures of 30 GPCRs (as of 2016) in active and inactive states, bound to agonists, inverse agonists and antagonists have provided a wealth of knowledge as to the factors controlling ligand recognition and activation steps (Audet & Bouvier 2012) (Xiang et al. 2016)

Several features of these new structures were important to the understanding of ligand recognition: the extreme variability of the ECL2 loop, the interaction of the ligand within the binding pocket, and the role of the overall structure in ligand recognition.

The second extracellular loop (ECL2) can be thought of as acting as a 'lid' over the binding pocket and shows extreme variability, with receptor-specific folds which form the entrance to the binding site within the receptor bundle. These variations result in a number of ways for the ligand to approach the binding pocket, for instance the S1P1 receptor ECL2 seems to seal off the binding pocket allowing only the lipid agonist S1P access to the binding pocket between the TM1 and TM7 helices in the plasma membrane (Hanson et al. 2012).

One of the most surprising results to come out of the structure 'explosion' was the multiple different ways in which the ligands interacted with the binding site when bound. In the β2AR for instance, the ligands bind within the predicted, deep binding pocket, parallel to the plane of the membrane but in the A2aAR, both agonists and antagonists bind perpendicularly within the binding pocket (Doré et al. 2011). This is unlike the chemokine receptor CXCR4 antagonist IT1t, which binds to the receptor on the surface between TM1, 3, and TM4, under ECL2 (Wu et al. 2010).

This diversity of ligand-receptor interaction also demonstrates that ligand specificity is not merely determined by the residues within the binding pocket but also the overall receptor architecture (Audet & Bouvier 2012). Witness for instance the high degree of overlap between the residues involved in the aminergic binding pocket of the adrenergic receptors β2AR and β1AR (Warne et al. 2008) and yet their very different functional selectivity. Some authors have proposed that this selectivity arises from conformational change caused by the ligand itself (Wacker et al. 2010), while others suggest that the ECL plays a role (Audet & Bouvier 2008) (Warne et al. 2008). The same binding pocket similarity with different ligand selectivity is demonstrated in the structure of the delta-opioid receptor (Granier et al. 2012), where the residues close to but not involving the binding pocket were shown to have a role. A role for residues not in direct contact with the binding pocket was also shown for the orphanin receptor (Thompson et al. 2012), these residues may have their effects via large pocket shaping and water coordination. A similar range of sites affecting ligand binding have been shown for ORs. (Mainland et al. 2014b)

The extreme variability in ligand selectivity between other class A GPCRs even though they share a great deal of sequence homology, should give pause to any extrapolations to the olfactory receptors.

As Bockenhauer et al (Bockenhauer et al. 2011) state: "the detailed molecular mechanisms by which ligand binding modulates GPCR activity remain poorly understood". They exist in a complex, dynamic environment with interactions between water, lipid membranes, ions and ligands; moving through multiple conformational states (Frauenfelder & Parak 1988). The available evidence is that they are not simple binary switches but rather act as summating 'integrators of information' (Bockenhauer et al. 2011) or 'microprocessors' (Kenakin 2009), constantly moving between the active and inactive

states, even in the absence of ligands. Ligand binding shifts this equilibrium one way or another depending on its role as agonist or antagonist. This stabilisation then increases the G protein-mediated downstream signalling. It may be that the functional selectivity arises from ligands stabilising different states and therefore different signals (Vilardaga et al. 2005).

The understanding of GCPRs overall is improving, our understanding of olfactory receptor function is even worse, lacking as we do any knowledge of the tertiary structure of even one of the receptors. The best that can be said is that nothing in the current literature forbids a vibrational mechanism in an olfactory receptor or other GCPR, although none has been demonstrated (to my knowledge this has not been investigated outside of olfaction).

## Signal Transduction in the Olfactory Sensory Neuron

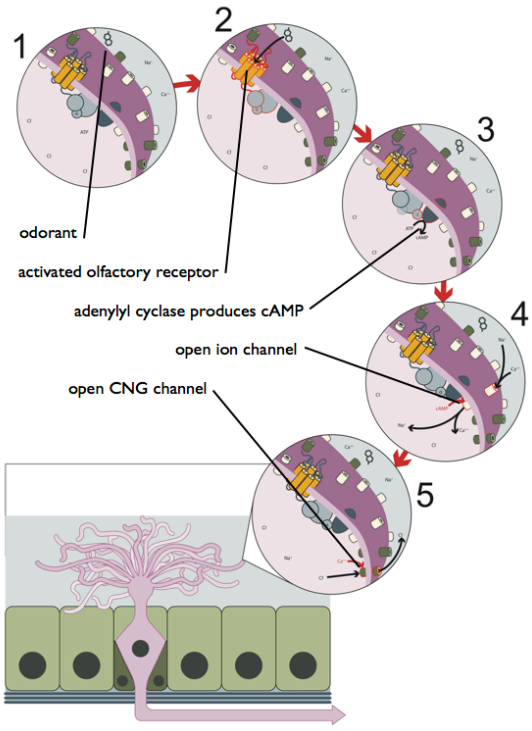
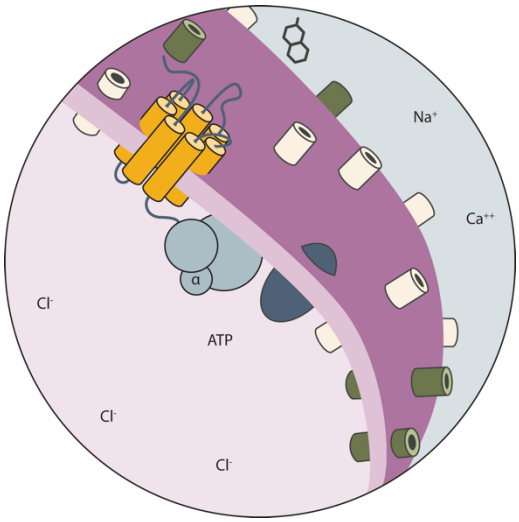



*Figure 18: Signal transduction steps at the membrane: 1: the odorant approaches the receptor. 2: the odorant binds and activates the receptor and G-protein. 3: the α-subunit stimulates adenylyl cyclase, producing cAMP. 4: CNG channels are opened, causing $Ca^{++}$ influx. 5: chloride channels are gated by $Ca^{++}$ allowing $Cl^-$ efflux and increasing depolarisation.*

Whether by one of the mechanisms described for other G-Protein coupled receptors or a vibration-detecting mechanism, the recognition of an odorant by the receptor is only the first in a chain of steps to depolarisation and the transmission of signal to the brain. Initial steps occur at the ciliary membrane with most of the actors being bound to the cell membrane. All of the units described below: ORs, $G_{olf}$, Type III Adenylyl cyclase (Menco et al. 1992) and CNG are found in much higher concentrations in the distal parts of the olfactory cilia. (Menco 1997)




The resting system consists of an olfactory receptor bound to a G-protein including the α-subunit on the cell membrane which is at the standard resting potential.

*Figure 19: The resting system*

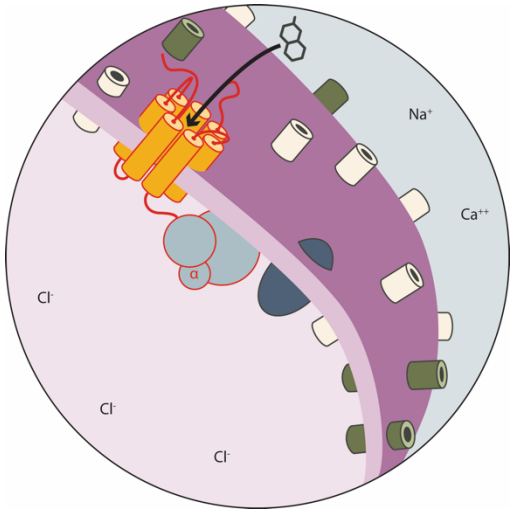



*Figure 20: Activation of the receptor*

The primary recognition step is binding and activation of the receptor. Whatever the method of activation, the olfactory receptor, once it 'recognises' the odorant, activates the GTP binding (D. Jones & Reed 1989)G Protein specific to the olfactory process: $G_{olf}$ (Belluscio et al. 1998) which then releases its α-subunit. The alpha subunit, through an as-yet unidentified process, stimulates the membrane-bound type III Adenylyl cyclase.

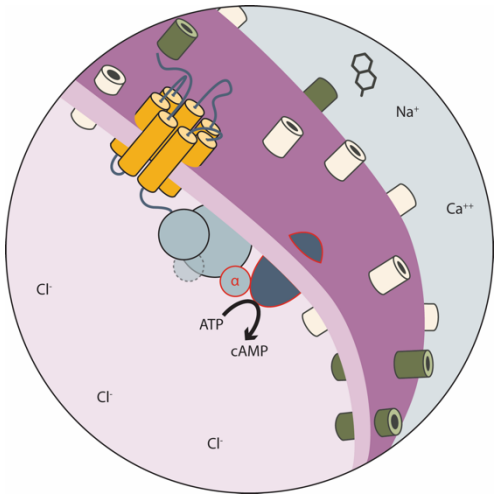



*Figure 21: Gα subunit activates ACIII*

Adenylyl cyclase (ACIII) forms the second messenger cAMP from ATP and it is the increase in cAMP that gates the first of two transduction channels (Nakamura & Gold 1987): the Cyclic Nucleotide-Gated channel, CNG. ACIII knock-out mice have been shown to have no recordable olfactory signals on odorant presentation, compromising their odour-dependant learning. (Wong et al. 2000)

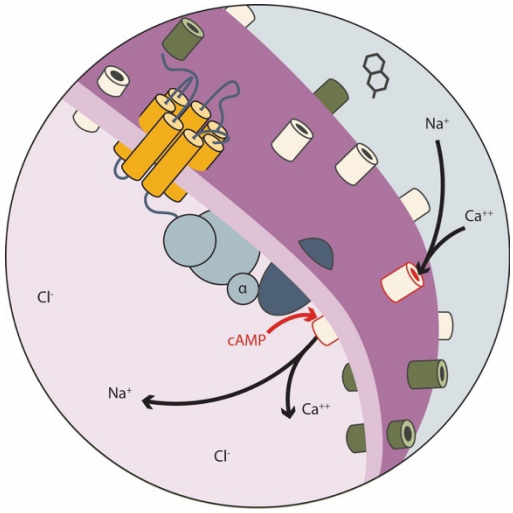



*Figure .22: Calcium channels gated*

CNG allows an influx of calcium and sodium. As well as being part of the inward depolarising current as a charge carrying cation, calcium modulates many of the parts of the intracellular machinery. Almost paradoxically it both excites and inhibits the olfactory response. (Matthews & Reisert 2003) It has been strongly demonstrated that this ion is responsible for the well-known olfactory phenomenon of adaptation, where the nose becomes "used to" constant smell stimuli after a while. (Menini 1999) (Kleene 1999).

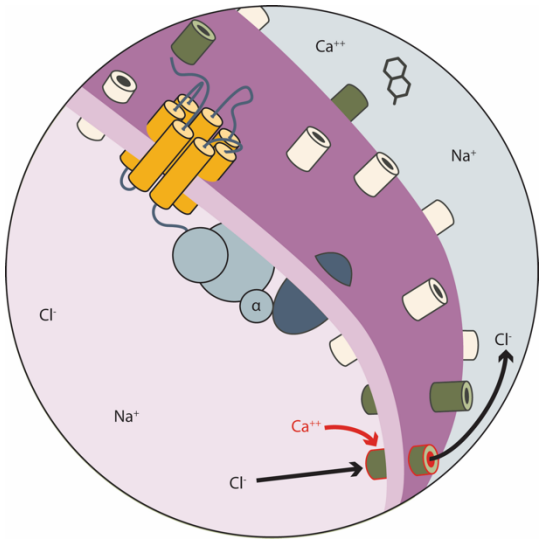



*Figure 23: Opening of chloride ion channels*

Calcium ions bind directly to another transduction channel: a $Ca^{++}$ - gated Chloride ion channel which allows $Cl^-$ to exit the cell causing further depolarisation. (Kleene & Gesteland 1991) This provides a low-noise non-linear amplification of the depolarising signal. (Lowe & Gold 1993) The molecular

identity of this channel remains a mystery.

(Kleene 2008)

The depolarisation signal passes via saltatory conduction in the standard way up through the cribriform plate to the olfactory bulb, to a very specialised structure within, the olfactory glomerulus.

## Higher Centres of Olfactory Signal Processing

### The Olfactory Bulb

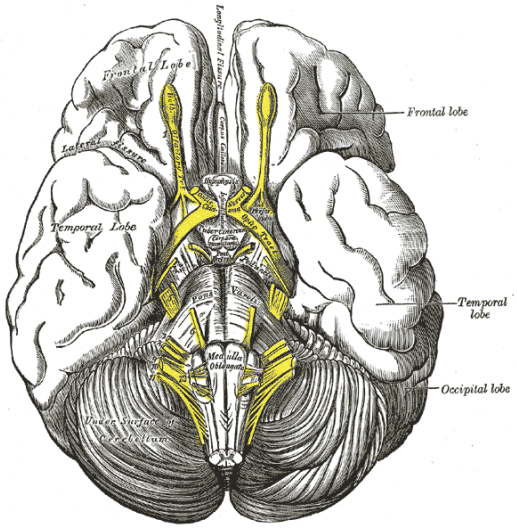



*Figure 24: The base of the brain showing the cranial nerves in yellow. Henry Vandyke Carter from Gray's Anatomy (1918) Anatomy of the Human Body Plate 724*

The olfactory bulb is an enlargement at the rostral end of the olfactory tracts, lying on both sides of the crista galli and contains a sophisticated primary processing system for the incoming olfactory signals, perhaps similar to the first order processing of the retina.

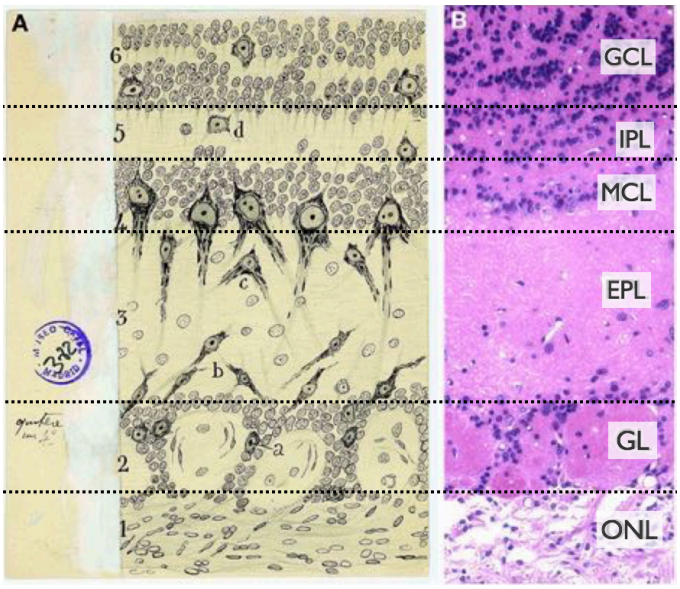



*Figure 25: A comparison of Cajal's original drawing and modern imaging of the layers of the Olfactory bulb. (A) Original Cajal drawing of the frontal section of the rabbit olfactory bulb (Ramón y Cajal, 1901). (1) Nerve layer (ONL). (2) Glomerular layer (GL). (3) External plexiform layer (EPL). (4) Mitral cells layer (MCL). (5) Inner plexiform layer (IPL). (6) Grains and white matter layer (GcL). (a) Peripheral tufted cells; (b) middle; (c) internal (d) short axon cells. Cajal Legacy (Instituto Cajal, CSIC, Madrid, Spain). (B) Olfactory bulb mouse coronal section of hematoxylin and eosin nuclei staining showing the different layers described by Cajal in (A). from (Figueres-OÃ ate et al. 2014)*

The bulb in humans has a laminar structure in common with most other vertebrates. It was first described by Ramon y Cajal (Ramon 1911) (see Figure 25) in 1911. Six layers are described (Smith et al. 1993): the olfactory nerve layer, glomerular, external and internal plexiform layers separated by the mitral cell layer, and finally a stratum containing just granule cells.

Once in the bulb, the neural axons trace a path in the olfactory nerve layer to one of between 2000 (Smith et al. 1993) and 8000 (Meisami & Bhatnagar 1998) structures, known as the glomeruli, within the glomerular layer (Ressler et al. 1994) (Figure 25). These

small spherical territories are conserved across the olfactory systems of vertebrates and invertebrates and seem to be the network-level representation of each olfactory receptor, as explained below.

**The Olfactory Glomerulus**

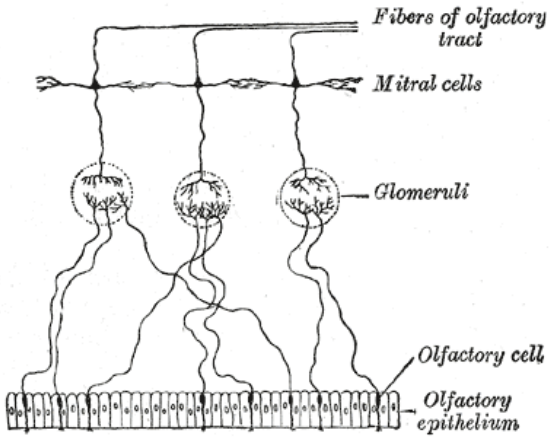



*Figure 26: Plan of olfactory neurons: (OSNs, glomeruli and mitral cells) by Henry Vandyke Carter: Grays Anatomy (1918) Anatomy of the Human Body, Plate 772*

The glomerulus is the brain's way of understanding the activation of olfactory receptors, only the olfactory sensory neuron expressing a specific type of receptor will grow to, and synapse within, a given glomerulus. That is, any given glomerulus only has afferent OSNs expressing one olfactory receptor, if there is any neural activity in the glomerulus, it is the comes from activation of a multiple cells all bearing a single receptor. This property can allow the upstream neural processing to remain fixed whilst the downstream OSNs can die off and be replaced.

The unique relationship of olfactory receptor to olfactory sensory neuron to glomerulus has been described as underlying a massively parallel process of "molecular feature detecting units" (Mori et al. 2006) which is utilised by the higher olfactory systems to produce a conscious perception of smell, allowing plasticity in the higher centres, learning

and association, whilst allowing continual regeneration of the OSNs exposed to constant environmental assault.

In mice, the activation of one glomerulus can be perceived even within an intense odour background and can transmit information using identity, information and temporal coding cues (Smear et al. 2013). It is known that different odorants are distinguished by different patterns (Sharp et al. 1975) (Johnson et al. 1998) (Xu et al. 2003), of activation within the olfactory bulb, but to what extent these patterns are recognised in time, space or both is still disputed (Leon & Johnson 2009). These odour maps - the spatial representation of the relative activation of receptors, have been shown in honeybees, moths, flies, zebrafish, rats, and mice (Mori et al. 2006), but not (yet) in humans. Similarly the arrangement of the glomeruli within the bulb is not understood, but doesn't seem to be related to molecular identity (Soucy et al. 2009).

The olfactory glomerular layer and the bulb more generally is the site of first order processing of the incoming stimulus (Shepherd 1974), with rich interconnections within and between the glomeruli (Gómez et al. 2005) (Kratskin & Belluzzi 2003) (Halasz & Shepherd 1983) (Kosaka et al. 1998). This processing has been shown to preserve identity information over varying concentrations (Schoppa 2009), before being passed to the higher centres via the olfactory tract.

**The Olfactory Cortex**

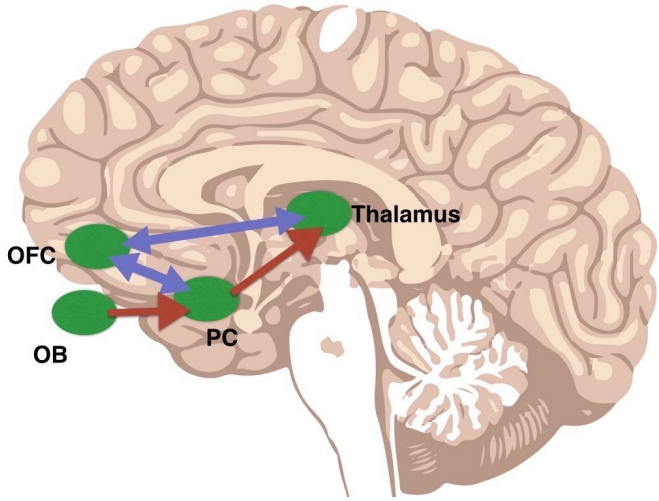



*Figure 27: Signal pathways between olfactory bulb (OB), piriform cortex (PC), orbitofrontal cortex (OFC) and thalamus, after Wilson 2012 (Wilson 2012)*

The lateral olfactory tract contains between twenty and fifty 'homotypic' mitral and tufted neurons from each glomerulus in the OB as they pass posteriorly to the olfactory or piriform cortex (PC). There are many synapses with other neurons throughout the tract, most also passing into the cortical structures (S. Ghosh et al. 2011). In mice, there are sites within the olfactory cortex which retain the chemotopic map information and other areas which reorder the pattern, with great diversity between individuals.

As in other special senses there is centrifugal input from the higher centres, with cognitive states such as expectation of odour (Zelano et al. 2011) or recall (Mainland & Sobel 2006) of an odour associated with activity within the anterior PC. This requires that blinding and setting are important to control in any psychophysical experimental procedures.

Gottfried and co-workers (Howard et al. 2009) demonstrated that patterns of activation on fMRI in the posterior piriform cortex (PC) were likely to be how odorant smells

were stored and that the right orbitofrontal cortex (OFC) is vital for the conscious appreciation of smell (W. Li et al. 2010).

Even this necessarily brief overview gives some idea of the complexity of the human olfactory system, the gaps in our knowledge of it and the caveats which must apply to any experiments undertaken, especially if those experiments attempt to use the system as a whole to understand the function of a part, such as the olfactory receptor.

## Rationale for This Thesis

This project is an attempt to improve the evidence base for the vibrational hypothesis in humans. I have pursued two strands of experiment. The first is to use a psychophysical approach: using humans as test subjects, can simple olfactory tests probe the relationship between vibrational characteristics and odour? This may be characterized more as a attempt at the falsification of the "shape" hypothesis with a human as an experimental model: if at least one human can be shown to perform a feat which a theory cannot explain, but another can, then the second theory should be preferred. This is not the same as requiring that all humans can perform the feat.

The second is to attempt to identify the olfactory receptor responsible for the perception of the probe molecule(s) to allow further investigation of its properties.

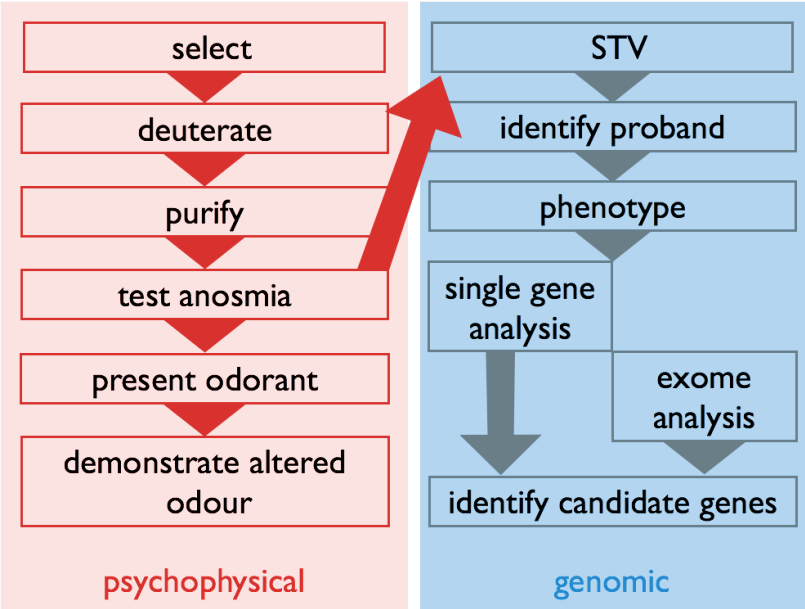



*Figure 28: Dual investigation strands, psychophysical and genomic, of the project and their interaction.*

Both of these experimental strands rely on the ability to vary the vibrational modes of a molecule without changing its shape. Deuterating molecules was previously a rather difficult and time-consuming process but with recent chemical advances using a catalytic process it has become simpler (although not simple), aiding this project enormously. With deuterated molecules it is possible to probe the system with agonists which vary by weight (and therefore, vibrational spectrum) and not by shape; if there is a predictable and reproducible variation in output between the two probe molecules, then that system is detecting something other than 'shape'.

## Aims and Objectives

### Aims

The aim of this thesis is to demonstrate role of molecular vibration detection in human olfaction by demonstrating a change of odour quality perceived by humans in molecules in which the vibrational, but not shape, characteristics of a molecule have been altered. Once this alteration of odour was demonstrated I attempted to identify a possible

responsible receptor to facilitate further investigation of whether this is indeed a receptor-level phenomenon.

**Objectives**

The objectives were to:

A. Demonstrate the altered odour of isotopically substituted common odourants in humans (psychophysical experiments)

B. Identify the olfactory receptor responsible for the detection of the odourant identified in A (genetic experiments)

## Thesis architecture:

In this chapter, I introduced the vibrational hypothesis of olfaction, review its history and development to the modern era and critically analyse its evidence base. I then reviewed the literature for GCPR activation as a family and summarised the current knowledge of the olfactory system in humans with special attention to processes which may modulate the structure-odour relationships.

In Chapter Two, I present the methods for investigating the psychophysics of olfactory perception using molecules predicted to have an altered odour by the vibrational hypothesis. In the second, genetic, strand of the thesis I review the methods for phenotyping and genotyping three families with an inherited anosmia to musk.

In Chapter Three, I report the results of the first set of three psychophysical experiments using deuterated molecules.

Chapter Four gives the results of the second strand of the thesis, examining the phenotype and genotype, using both single gene and olfactory exome-wide techniques.

In Chapter Five, I discuss the findings presented in chapter 3 and 4 with strengths and weaknesses, as well as interpretation of the results in light of the wider literature. I review what this thesis adds to that literature and the implications for further research.

In Chapter Six I draw the conclusions from the discussion and my findings.

# Chapter Two: Methods

## Chapter Synopsis

In this chapter I discuss methods of investigating the vibrational hypothesis in humans. I report the methods for two strands of investigation: the psychophysical and genetic, to support the objectives previously outlined. In the first strand I attempt to reliably demonstrate that there is a variation in odour quality between pairs of odorants whose molecular structure is so similar that it would not be expected to be altered under the dominant "shape" recognition paradigm. These are:

1. Produce "probe molecule " pairs with altered vibration but not shape by selecting and deuterating the appropriate odorant

2. Demonstrate an objective alteration of the odour after deuteration in multiple volunteers, controlling for contamination of samples, possible confounding odours, and olfactory ability; in a fully blinded experiment

I then characterise the methods for phenotyping an inherited specific anosmia for some musks in three families and explain the methods for genotyping these families in using both a single gene and exome-wide methods to identify a receptor which may be responsible for the anosmia therefore worthy of further investigation. These methods may be summarised as:

A. Identify an inherited specific anosmia for a characterised molecule in a proband family

B. Phenotype the inherited specific anosmia

C. Examine the genotype via Sanger sequencing of a known candidate gene for abnormalities which segregate with the phenotype

D. Examine the genotype via exome sequencing for abnormalities which segregate with the phenotype

## Introduction

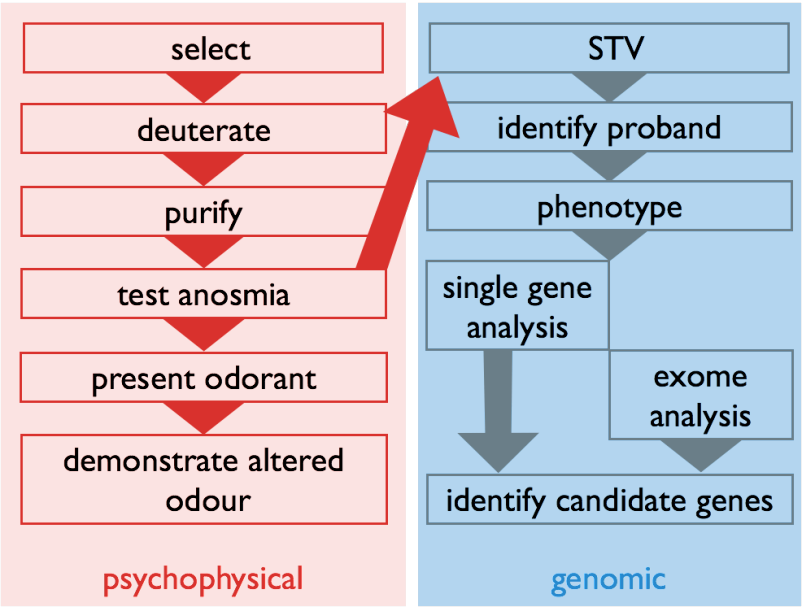



*Figure 29 Project outline - dual investigation strands, psychophysical and genomic, of the project and their interaction.*

The vibrational hypothesis is a quantum biological description of receptor function and its effect on odour. To examine it requires a demonstration that odours are altered in a way predicted by the hypothesis and that this alteration is a receptor-level event. The difficulty arises from the poverty of OR-ligand interaction data as discussed in the previous chapter as well as the limits of the technology to investigate quantum-level events in biological systems. Linking the psychophysical with the physiological in humans had only been possible for one odorant receptor at the beginning of this project (Mitchell et al. 1987), although more were discovered over the following years.

As shown in Figure 29, my goal in the first part of the project was to demonstrate that vibrational alteration resulted in odour alteration when stereo-chemical properties

were preserved, something that had not been shown in vertebrates before. To do this, I had to choose the right probe-odorant pair.

Once I had demonstrated that vibrational characteristics altered the odour percept at an organism level, various techniques could be used to identify the receptor involved and examine it more closely.

By a stroke of serendipity, none of the more complex receptor-deorphanisation techniques were required as we identified a previously documented (Baydar et al. 1993) specific threshold variant (STV) in some of our volunteers. This specific anosmia to musks, especially to the musk Galaxolide, allowed an exome-wide approach to the identification of possible causative genomic DNA variations.

Armed with these genetic variants it would then be possible to express any ORs identified and examine these for variable activation by the probe molecules or other ligands. Alternatively, if genomic OR variants were not robustly associated with the phenotype, but other genes were, that might point to a perireceptor event if the gene discovered were that of an enzyme, for instance. This would go some way to providing an alternative explanation of all of the findings presented here, should the vibrational hypothesis be disproved.

## Investigating the Vibrational Hypothesis of Human Olfaction

There are several possible methods of investigating the specificity of the olfactory receptor. In this project, I have used in-vivo psychophysical experiments in humans as a first step, to demonstrate that this is a real phenomenon in human olfaction and not just an interesting molecular-level detail. I then go on to examine the genetics of the putative

olfactory receptor associated with the psychophysical findings in an attempt to identify the responsible OR. I will discuss these techniques here.

## Psychophysical test methods

First coined by Gustav Fechner in his "Elements of Psychophysics" (1860, English translation: 1966); psychophysics is the study of the relationship between objective physical stimuli and the percepts elicited by them in the human mind. The underlying principles have been used to investigate all sensory modalities include non-sensory percepts such as price and value. (Kamen & Toman 1970). Often the methods are used to determine the structures underlying the percepts, to see the mechanisms of the system by the way it fails.

Turin's model predicts quite a few psychophysical findings, several of which were at least partially tested by the Keller and Vosshall work in 2004 (Keller & Vosshall 2004). The primary claim, and one which has the greatest opportunity for clinical testing, was that the odour of a molecule should vary depending on its suite of molecular vibrations. A simple approach to this would be to smell isotopes of a molecule (the easiest being deuterated and non-deuterated versions) and say whether they were different or not. This is a classically psychophysical approach to the problem and the most biologically relevant: structure-odour relations are about the odour, after all.

## Test Methods

The food and fragrance industries have developed the methods for psychophysical detection of perceptual differences to an advanced level, often requiring sophisticated statistical analysis. Although these subtle distinctions can be useful the vibrational hypothesis should result in rather obvious alterations in odour profiles if it is to be interesting. This allows 'cruder' psychophysical tests as the effect size should be large enough to be easily detected.

There are many possible psychophysical techniques for investigating the ability of the olfactory system to distinguish between olfactants.

Green and Swets (Green & Swets 1988) laid the foundations for signal detection theory and divided signal-detection tasks into three types: yes-no, rating and forced choice.

That is, the subject is presented with a signal task and must decide whether it is "signal" or "noise" and responds with either the yes-no, a rating of confidence in their decision, or to a forced choice between two or more members of a group.

Suprathreshold similarity is an example of a yes/no trial, using this technique the subjects are required to rate the similarity of odorants in a pair: A and B. The members of the pair are randomised between the two odorants being investigated so that for any one trial the comparison may be between "A" and "B", "B" and "A", "A" and "A" or "B" and "B". The randomisation has to be with replacement or the proportional chance of choosing the second of the pair is altered. The subject states whether the pair is the same or different and is marked as correct or not. Statistical analysis is simple as the chance of success is 50%.

In the two-alternative forced choice test (2AFC), the subject is trained by exposing them to one or the other of the test odorants initially and asking them two identify that odorant from one or more subsequently presented pairs.

Using three odorants (3AFC) at a time removes the need for a training set and allows the subject to select the "odd one out" between the three. The chances of this are only 33% and so fewer trials are needed to demonstrate significance. Randomisation is more difficult, as is blinding, as the experimenter has to know the identity of the samples and randomise the third sample to one or the other of the test odorants although it is

possible to then randomise the presentation of the samples such that the assessment in double-blinded.

The R-index has been used to demonstrate threshold variants (Jaeger et al. 2010). It is a variation on the rating task where the panellist is required to judge the relative intensity of a signal and also rate their confidence in that judgement (Robinson et al. 2005). It has been shown to be an efficient way of determining thresholds compared as opposed to an ascending or descending limit but requires more sophisticated analysis.

Due to the simplicity and robustness of the yes/no trial for supra-threshold similarity, I chose to use this for the first experiment. In the second, musk detection experiments, technical limitations necessitated a two-alternative forced choice regime, as discussed in that chapter.

Sources of Psychophysical Error

Psychophysical techniques cannot demonstrate that inelastic electron tunnelling is taking place within the receptor. All they can do is determine that odorants whose "shape" is "the same" smell different to humans if their vibrational characteristics are different.

However, even if it is possible to demonstrate this difference, there are some caveats to this apparently simple test, some of which can be controlled through experimental design, and some cannot.

Differential Chemistry

Deuterium isotopes have almost the same size and shape (within thermal "smearing" at room temperature) as their hydrogenated counterparts but they can have very different chemistries. The degradation products of the chemical just left in a sample tube can be very different and they can cause marked changes in the odour of unpurified compound. The presence of large number of enzymes within the olfactory mucus layer only compounds this

effect. Although the enzymatic process is likely to be slow it may be possible for the separate chemistries to lead to two significantly different populations of degradation products (Schilling & Schilling 2009). Additionally, because of the extremes of human olfactory sensitivity, any other contaminant, even in very low concentrations could alter the odour of a sample. This necessitates fresh samples of the very highest purity, which are really only possible with preparative gas chromatography.

Isotope binding effects are well described (Schramm 2007), isotopic variation alters the bond vibrational environment and therefore the binding to an enzyme or receptor. These binding isotope effects, "BIE"s, do not seem to be predictable for any given reaction: they can be marked or negligible and can potentiate or inhibit the reaction they alter. Unfortunately, there does not seem to be a way of predicting these effects, especially given the lack of knowledge of the structure of the OR, I was unable to control for this within these experimental methods but identifying the putative receptor would allow finer control of the experimental milieu once expressed in a heterologous system.

Differential Mechanics

The force of sniffing the odorant may vary between trials and this may alter the percept (Teghtsoonian et al. 1978) (Mainland & Sobel 2006). This should be a source of noise error rather than bias and can be controlled by technique (using an olfactometer or atomiser for passive presentation) or by analysis: noise should be averaged out in multiple trial tests), hence the large number of rounds in the acetophenone experiments.

Human Variability

Smell in untrained humans is a notoriously unreliable sense (Laing & Glemarec 1992) (it is not much better in professionals) (Livermore & Laing 1996). Using volunteers with no training in olfactory assessment risks missing a detectable signal (a shortcoming of

the Keller-Vosshall experiments (Keller & Vosshall 2004)), hence the use of trained perfumers in the first set of experiments. Additionally, each human nose is highly variable in the receptors it expresses. It has been shown that the pattern of pseudogenisation is highly variable within and amongst different ethnic groups (Menashe et al. 2003), so we should expect a range of specific anosmias, especially in the untrained smeller. (Although some specific anosmias can be overcome with training (Wysocki et al. 1989)). For this reason, I excluded those with an STV to musks in the musk trial.

## Probe Molecule Selection

Choosing the correct probe odorant is key to successful demonstration of alteration in the odour of a molecule when deuterated. There are many possible candidates, but the ideal odorant would be:

- cheap
- conserved surface topology with vibrational alteration
- safe (a recognised perfumery ingredient)
- pleasant to smell
- easily perceivable in low concentrations
- slow to habituate
- act on a very small subset of the available receptors

No such perfect odorant exists, of course, but there were three possible groups of probe molecules: deuterated molecules, metallocenes and silica analogues.

### Deuterated Molecules

Isotopic substitution of deuterium for hydrogen in organic molecules alters the vibrational modes without appreciably altering the surface topology or "shape" (Block, Jang, Matsunami, Sekharan, et al. 2015). Because the vibrational frequency of a molecular bond is a product of the mass of the molecules in the bond, isotopes of a molecule

have differing vibrational spectra, not merely in one frequency, but since the whole is a complex harmonic system the variation can be marked. The easiest isotopic change is deuteration, the substitution of hydrogen with the heavier deuterium atom. This obviously changes the contribution of the C-H bond in most organic molecules as can demonstrated for acetophenone in Figure 30.

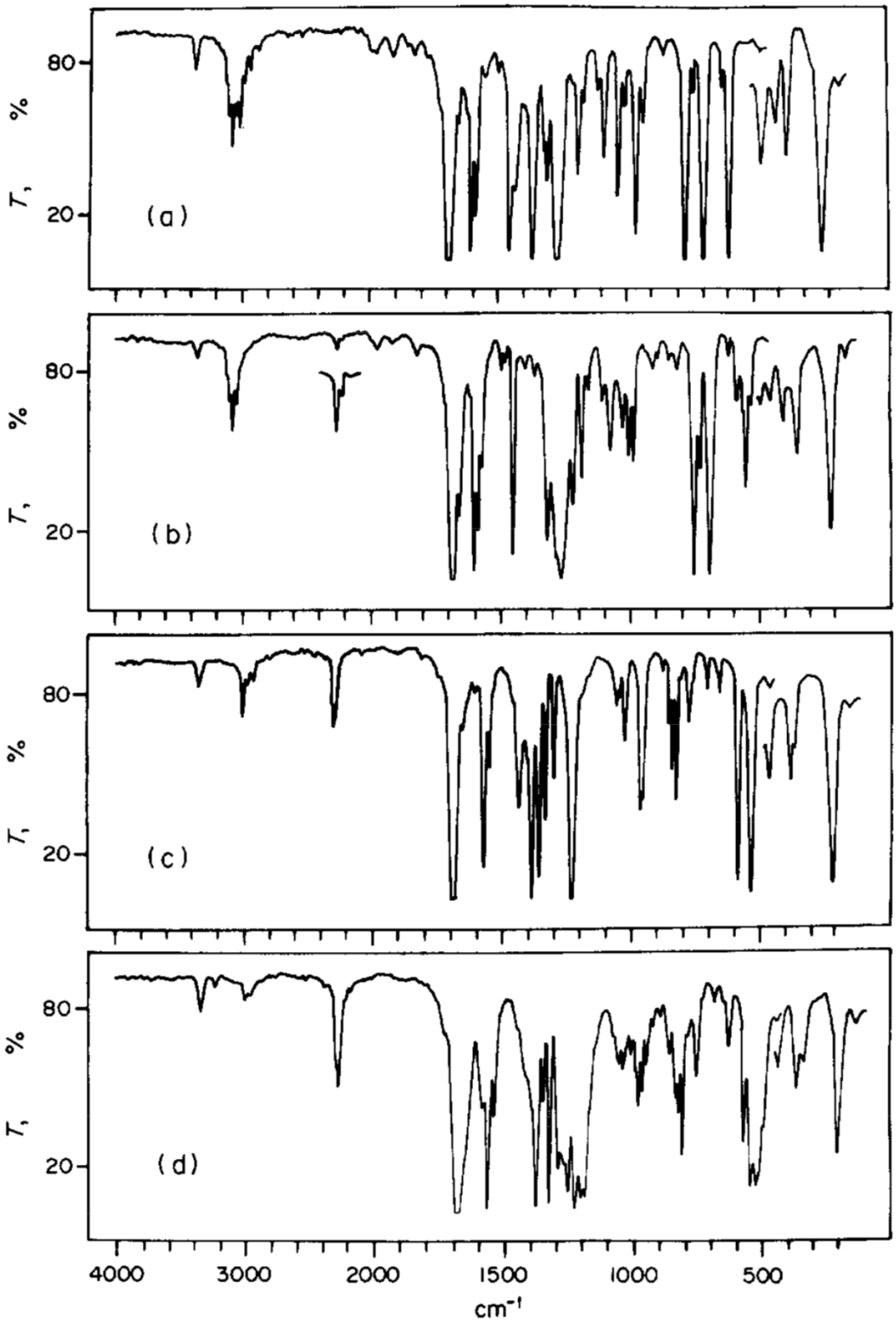



*Figure 30: Figure 1 from Gambi et al (Gambi et al. n.d.) The IR spectra (from 4000-130cm$^{-1}$) of (a) acetophenone-$d_0$, (b) acetophenone-$d_3$, (c) acetophenone-$d_5$, and (d) acetophenone-$d_8$, in the liquid-phase, the y-axes are the relative intensities. The loss of bands in the 1200-800 cm$^{-1}$ range in the perdeuterated (lowest graph) is readily apparent.*

The initial approach was to use the first three criteria for selecting the odorants below:

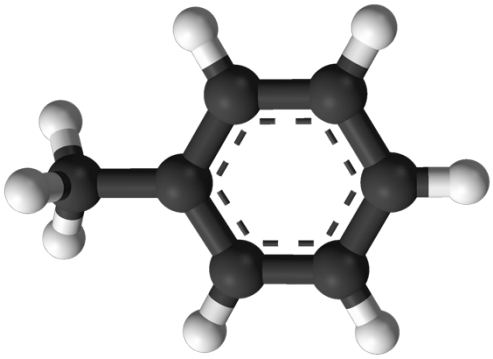


*Figure 31: Toluene, Ball-and-stick model of the toluene molecule, $C_7H_8$, as found in the crystal structure. X-ray crystallographic data from J. Chim. Phys. Phys.-Chim. Biol. (1977) 74, 68-73. Model constructed in CrystalMaker 8.1. Image generated in Accelrys DS Visualizer. From Wikipedia Commons (user: Benjah-bmm27).*

**Toluene -** (IUPAC name - Methylbenzene) (Figure 31) a mono-substituted benzine derivative. Used as a common solvent. Unpleasant solvent odour and used as a substance of abuse, toxic in large doses.

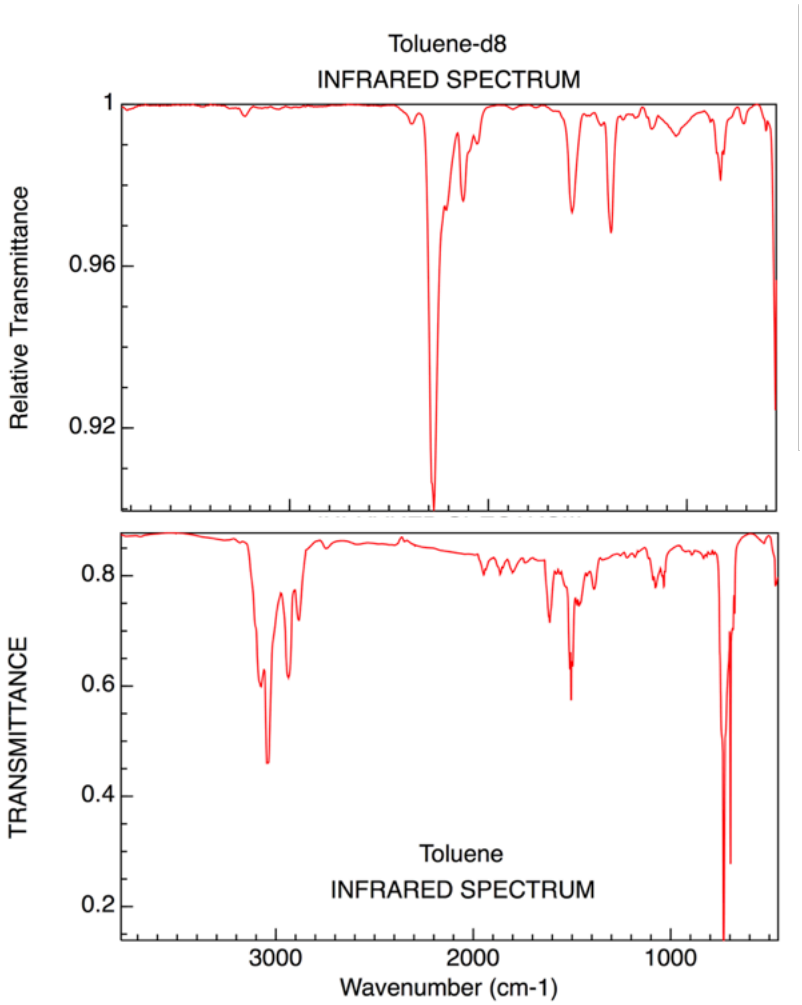



*Figure 32: Comparison of infrared spectrum of toluene and toluene-d8. Note that the y-axis scales are not the same as the upper plot is of relative transmittance. Images from NIST Standard Reference Database 69: NIST Chemistry WebBook*

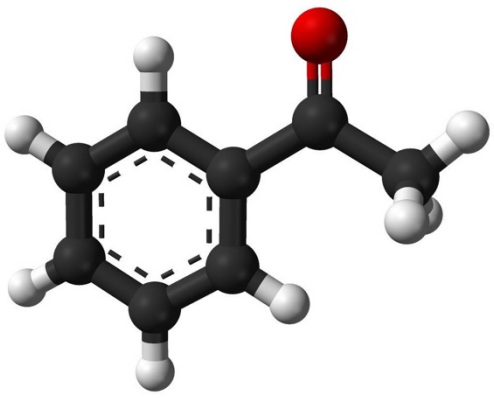


*Figure 33: Acetophenone, Ball-and-stick model of the acetophenone molecule, PhCOMe, C8H8O, from the crystal structure. X-ray crystallographic data from Acta Cryst. (1973). B29, 1822-1826. (Tanimoto et al. 1973) Model constructed in CrystalMaker 8.1. Image generated in Accelrys DS Visualizer. From Wikipedia Commons (user: Benjah-bmm27)*

**Acetophenone** – (IUPAC name: 1-phenylethanone) (Figure 33) is the simplest aromatic ketone, occurs naturally in fruits and vegetables such as apple, apricot, cauliflower and banana. A "fruity" "pleasant" smelling common investigative odorant that has already been shown to be distinguished in deuterated and non-deuterated forms in Drososphila. (Franco, Turin, Mershin & Skoulakis 2011b) The difference in the isotopes is supposed to be so striking that it is named as the central supporting fact in Turin's 1996 paper. (Turin 1996)

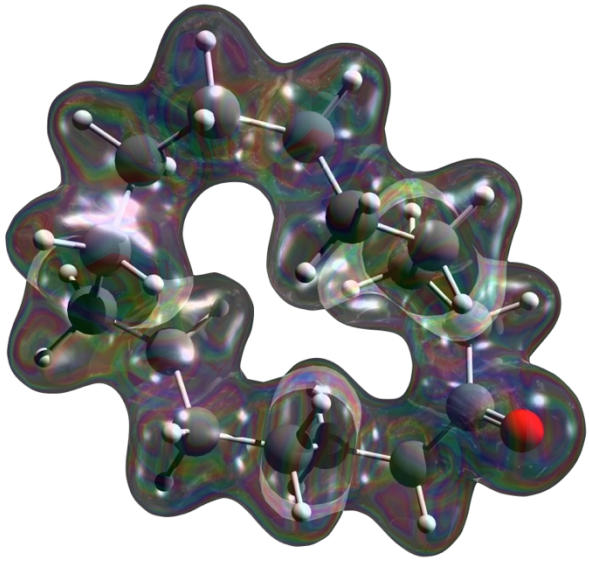


*Figure 34: Cyclopentadecanone (Exaltone®) inside its electron cloud. Structure calculated with Amsterdam Density Functional and rendered with Strata 3D. (courtesy L. Turin)*

**Exaltone®** (IUPAC name: cyclopentadecanone) (Figure 34) gave the highest yield and the fewest side products (a few percent, chiefly cyclopentadecane and cyclopentadecanol,

the latter easily removed by silica gel chromatography) on catalytic deuteration. It is in addition thermally stable and an extremely powerful odorant.

Calculated Raman spectra for the isotope pair are displayed in Figure 35 below. The area of obvious difference between 1300 and 1600 wave numbers is highlighted.

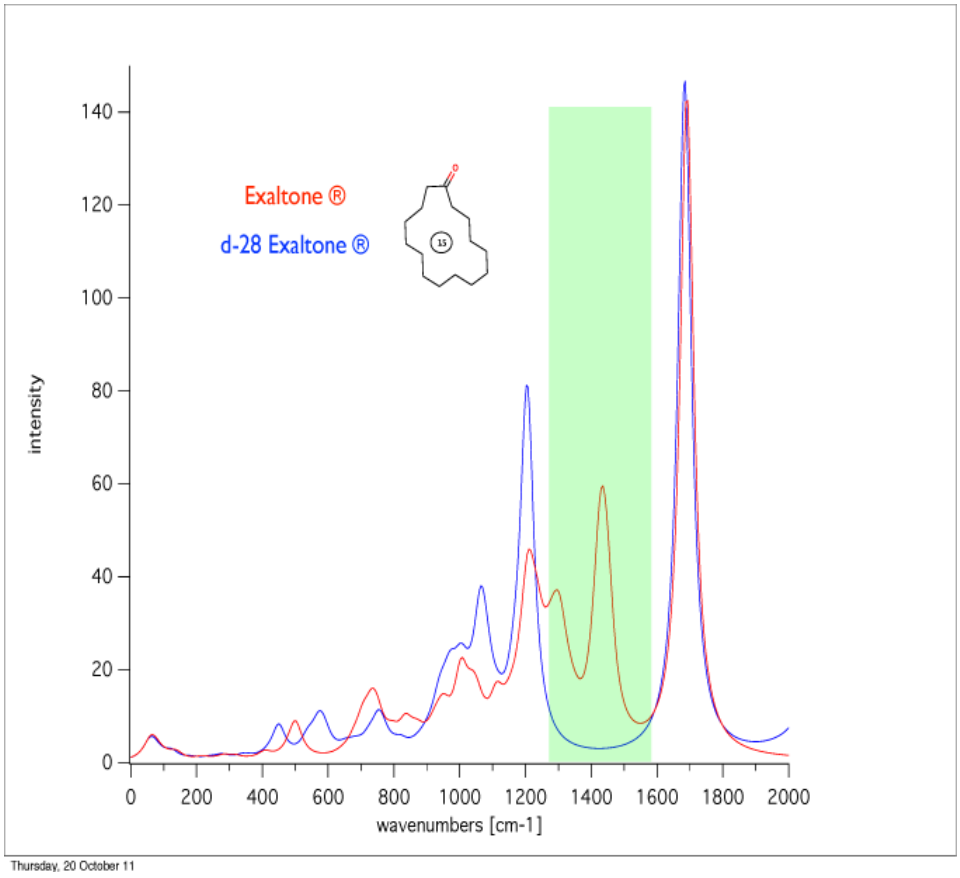



*Figure 35: Calculated Exaltone spectra for hydrogenated and per-deuterated isotopes (courtesy L. Turin). The difference in the spectrum between 1300 and 1600 wavenumbers is highlighted in green.*

Why Musks?

Musks are a good choice of target deuterated olfactants for several reasons: it is known that they do not habituate as quickly as acetophenone, as they are larger molecules they contain more C-H bonds which will be perturbed by H-D exchange, and they probably activate very few receptors, perhaps only one or two as explained below.

Deuteration changes the characteristics of the C-H bond. It seems likely that bigger molecules with more of these bonds might show a difference in odour character when the hydrogen was replaced with deuterium. Musks are among the largest odorants and typically contain 15-18 carbons and 28 or more hydrogens (Theimer 1982), as compared to 8 carbons and 8 hydrogens for acetophenone.

Given that the vibrational hypothesis is a receptor-level explanation of ligand activation, the ideal experiment to demonstrate the vibrational hypothesis would concentrate on just one receptor and demonstrate a variable activation of that receptor with molecules of the same shape but differing vibrational modes. Since there are probably a very small number of musk receptors, with musks we can create a "single receptor human" (by analogy to Richard Axel's "single receptor mouse" (Fleischmann et al. 2008)) and probe this receptor behaviourally. The evidence for this single, or at least extremely low number, musk receptor is from three independent sources: a correlation with molecular size and receptor recruitment, the well-documented specific an- and hyper-osmias to musks and structure-odour modelling.

Overview of the Musk Odorant Families

Musk molecules play an important role in this project and some background is useful. The group is named for the perfumery ingredients first obtained from the scent glands of the musk deer (*Moschus spp*) in eastern and southern Asia (Russia, China, Nepal and Tibet etc.) and are a common, almost ubiquitous, perfumery component. They are found in fine fragrances and in laundry detergents. Describing the characteristic "musk" scent is difficult and leads to sentences such as "They can be elegant, eclectic, eccentric, emotional, exciting, evocative, exotic, extreme, ecstatic, extravagant, energizing, or exalting in a perfume; but always in an erogenous way." (Kraft 2004a). The usual odour descriptors are "animalic", "sweet-powdery", "warm" and "sensual".

The first artificial musk, Musk Bauer, was synthesised in 1888. This was before the components of the musk deer ingredient had been identified. Musk Bauer was an accident, created by Albert Bauer whilst trying to create a better form of TNT. Other, similar so-called nitro musks were soon created, including musk ketone and musk xylene. These are no longer commercially available due to concerns about neurotoxicity and biodegradability. (Kraft 2004b)

These musks are representative of one of four types of musk structure, the nitro musks. The other structural groups are polycyclic, macrocyclic and alicyclic.

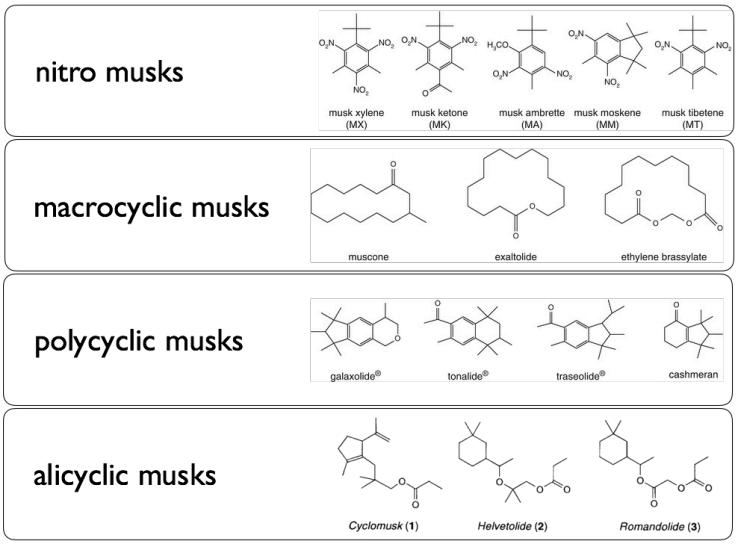



*Figure 36: Musk structural groups. Structures from (Chisvert et al. 2013) and (Eh 2004).*

All naturally occurring musks are macrocyclic, those from animals being ketones and those from plants being lactones (macrolides). Polycyclic musks (Galaxolide®, Tonalide®, Phantolide®) were the mainstay of commercially available musks but macrocycles (ethylene brassylate, Habanolide®) are now more common Typical structures are shown in Figure 36. Alicyclic or linear musks are relatively modern with the first commercially available "Cyclomusk®" coming on to the market in 1975 (Kraft 2004a).

In Turin's explanation of the ramifications of vibrational hypothesis in terms of odorant design (Turin 2002) discusses the musks at some length. He posits that a musk odorant must have vibrational peaks within four bands: 700, 1000, 1500 or 1750 (for Nitro musks) and 2200 cm^-1, but also shows that Galaxolide does not have a peak in the 700cm^-1 band. It is worth noting that here, he does not use the experimental IR spectra of the musks but rather the calculated spectra. This he estimated to give a margin of error of 0.8-6% in the calculated frequency of the spectral peak but does not mention the accuracy of prediction of a peak at all.

Twelve years later Maia et al (Maia et al. 2014) assessed two different methods for the calculation of musk infrared spectra and showed that even with the advances in technology there was still a difference between the two methods. What is more, even then the more accurate of the two methods was unable to show a correlation between the IR spectra of the calculated molecules and their "muskiness".

The other reason that musks are of interest is that they are probably detected by a relatively small number of receptors, based on human psychophysical studies and mouse receptor techniques, as discussed below.

The Similarity of Musk Odour

Although there are variations in tonality and the "facets" of the smell of the musk molecules there is an undoubted common musk odour which underlies the scent of all the widely differing musk molecules. For a long time it was been assumed that this would mean there is a common musk receptor which is activated by all of them as in Amoore's pre-receptor "primary odours" theory (Amoore et al. 1977).

This is of course not necessarily true, the percept might arise from two receptors with a similar glomerulus, or a common breakdown product produced by all the molecules

while in the olfactory mucus which stimulates another receptor, or perhaps a shared OBP which only facilitates the binding with certain receptors and therefore a learned similarity, but the evidence below supports the hypothesis that there a few highly-specific musk receptors in humans.

Human Musk STVs Fall into at Least Two Groups

Human musk anosmias have been described for over eighty years. Guillot (Guillot 1948) was probably the first to record his own musk anosmia, although it is likely that this was known to perfumers much earlier. He also suggested that this specific anosmia was a useful tool to investigate the olfactory system. Several musk anosmias have been described with Amoore being the first to document an STV to Galaxolide (Amoore et al. 1977). This paper noted that the anosmia for pentadecalactone did not co-vary with the anosmia for Galaxolide but did seem to predict the threshold for musk ketone.

A specific anosmia for pentadecalactone was described by Whissel-Buechy (Whissell-Buechy 1973), who demonstrated a simple Mendelian inheritance pattern characteristic of a recessive gene prevalent in subjects of Western European origin and absent in the African American subjects tested. This pattern conforms to that of the receptorfor β-ionone (Plotto et al. 2006), i.e. a high-penetrance determinant of the threshold for that odorant. This STV for Exaltolide (ω-pentadecalactone, a macrocycle) co-varied with that of musk ambrette, a nitro musk (i.e. anosmia for one predicted anosmia for the other) but the threshold for musk ketone, another nitro musk, did not. Interestingly, mouse OSNs in Nara et al (Nara et al. 2011) which demonstrated activity to Exaltolide, did not react to musk ambrette and vice versa. It is likely that there are at least two musk receptors, both of which can be stimulated by different nitro musks. The other possibility is that the two musk receptors for ω-pentadecalactone and musk ambrette are closely

genetically associated and more likely to be inherited together, whereas the musk ketone OR gene is not.

Avery Gilbert and Sarah Kemp (Gilbert & Kemp 1996) used a psychophysical two element forced choice test to investigate musk thresholds in 32 volunteers. Clustering the results demonstrated four groups: a group (n=12) generally able to detect all musks and the control non-musk odour (phenylethyl alcohol, PEA), a group with a generally poor ability to smell musks and PEA (n=16), a group (n=2) which was poor at detecting all odorants EXCEPT two musks: Exaltone (cyclopentadecanone) and musk xylol, and finally a group (n=2) which showed a similar insensitivity to all but Δ9-hexadecenolactone (ambrettolide) and tonalid. Interestingly this is an STV with hyper- and hyposmic components and the incidence for both of these hyperosmic subtypes was 6.25%, about the same as the 7% incidence for the anosmia demonstrated by Whissell-Buechy. A specific hyperosmia implies a likely hyper-functioning receptor in the same way an anosmia implies a non-functional one and the presence of two distinct groups argues for, again, two receptors.

Gilbert and Charles Wysocki in their analysis of the National Geographic Smell Survey (Wysocki & Gilbert 1989) had estimated the population prevalence of Galaxolide anosmia to be approximately 20 to 30%, whereas in a much smaller sample Baydar et al (Baydar et al. 1993) found 10.5% of women and 7.3% of men to be unable to detect Galaxolide at the maximal concentration. This is likely to be a severe underestimate of the population mean as the sample population were all fragrance company employees and therefore likely to be better than average smellers.

The mere presence of an STV doesn't confirm it to be genetic, there is no requirement for an STV to be inherited. Knaapila (Knaapila et al. 2012) demonstrated that although several other odorants they tested in a large group of twins did not have heritable thresholds

or valence, androstenone and Galaxolide did. They hypothesised that a genome-wide association study would demonstrate a variation in the olfactory receptor genes to account for this. The wide genotyping they first performed did not detect any association with OR genes, but once the known androstenone receptor OR7D4 was specifically genotyped, this was significantly associated with the STV phenotype. No candidate receptor was found to explain the Galaxolide STV.

The experiment only examined SNPs and not CNVs/indels as previously discussed, in addition the genotyping platform used had markers only within 38% of OR genes, so a SNP may still be a possible cause of the Galaxolide anosmia. In either case, higher resolution sequencing and better bioinformatic techniques would be more likely to find the genetic cause of the musk anosmia, nearly eighty years after it was first documented as a clue to the odour code

Kraft (Kraft 2004a) cast doubt on the likelihood of just one human musk olfactory receptor - although he also declared that dedicating more than three, or just under 1% of the human ORs to musks "seems a lot". He was able to show that using an olfactophoric method a great deal of structural variation between the musks could be ignored and that one receptor could potentially accommodate a great deal of the molecules.

All the strands of evidence indicate that there are a small number of highly selective musk receptors triggered only by musks, so an approximation of the "single receptor human" might be a possibility. All of this evidence relies on examining the whole human perception of odours, but work in mice demonstrates that these variations arise because of receptor differences.

Mouse Musk Receptors are Limited in Number

Although little was known about human musk receptors at the start of this project, mouse receptors were assumed to be relatively few in number, correlating with the human STVs outlined above. Understanding the mouse receptors sheds some light on what might be expected in the human,

Size vs Activation in Mouse Models

That musk receptors are low in number and narrow in range, at least in mice, is demonstrated by some work by Nara et al (Nara et al. 2011), who performed a large scale analysis of dissociated mouse OSNs from a group of C57BL/BJ6 mice. They exposed the OSNs harvested from the olfactory epithelium to a series of odorant mixtures and then one by one to the individual contents of those mixtures. 125 odorants were tested, grouped initially into thirteen mixtures by structural similarities (amines, aldehydes etc.).

Only sixteen OSNs responded at all to the musk mixture, containing eight musks (neither Exaltone® or Galaxolide were examined, unfortunately). Ten of these responded to at least one other mixture although there seems to be no pattern to the identity of the other mixture, see Figure 37.

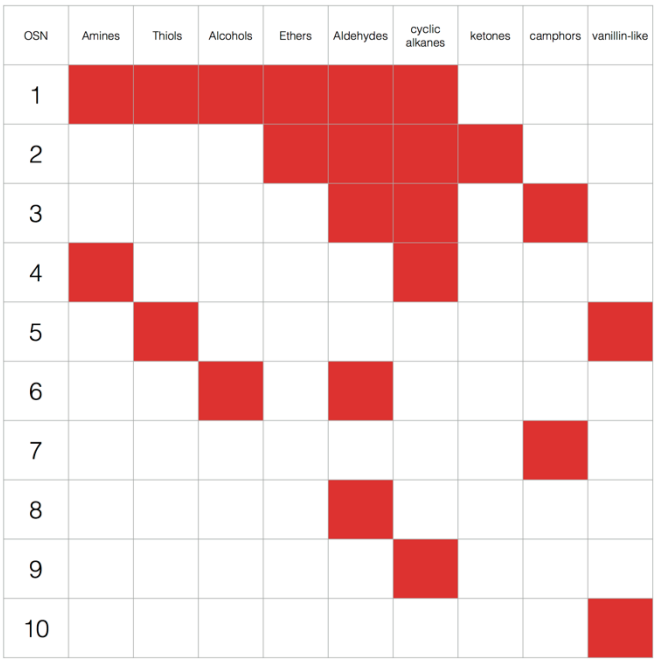



*Figure 37: Mouse OSNs reacting to multiple odorant mixtures in Nara et al (Nara et al. 2011) Cells are those which demonstrated activity to the odorant mixture are marked red. Redrawn from data from Figure 2 in (Nara et al. 2011) for all OSNs reacting to the musk mixture and at least one other (shown).*

The six neurons which had responded to the musk mixture alone, were further exposed to the individual musks one at a time shown in Figure 38.

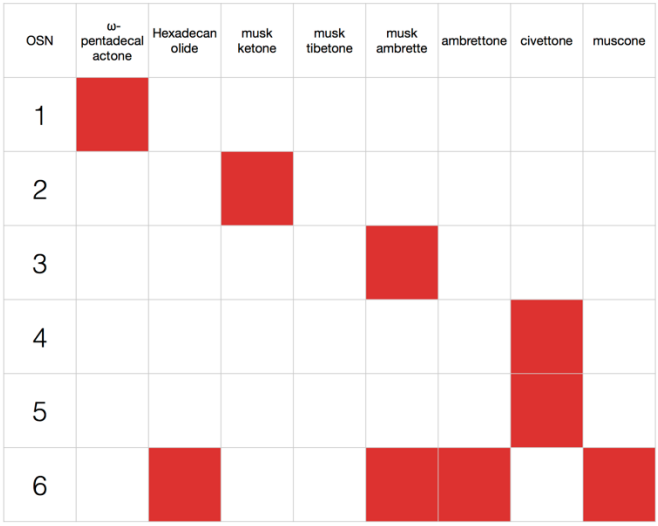



*Figure 38: Mouse OSNs reacting to only the musk mixture. Single musk exposure results. Cells are those which demonstrated activity to the odorant mixture are marked red Redrawn from data: detail from Figure 4. (Nara et al. 2011)*

Replotting of size of odorant vs number of receptors activated in mice from Nara et al. Figure 39 demonstrates a roughly linear relationship between odorant size (expressed as

logP water/Octanol) and number of receptors activated (Cometto-Muñiz & Abraham 2010b).

It is a well-known empirical rule of perfumery that the cut-off for odorant size is around 18 carbons, there are no larger molecules which are detectable by olfaction. This is not due to volatility, since heating an odourless 20-carbon compound will not make it smell (R. Duprey, president of the British Society of Perfumers, pers. comm.)

So, with 18 carbons as the upper limit and a relatively linear relationship between odorant size and receptor activation in the lower sizes, there is likely to be a rapid fall-off in receptor recruitment by the larger size molecules as their size tends to the upper bound. This implies that a molecule with a size at the upper limit probably stimulates only a few receptors. (Of course, it may be possible to have a linear relation with size up to the limit, with the largest molecule stimulating the most receptors, but that is unusual in a biological system and unlikely given the other evidence below.)

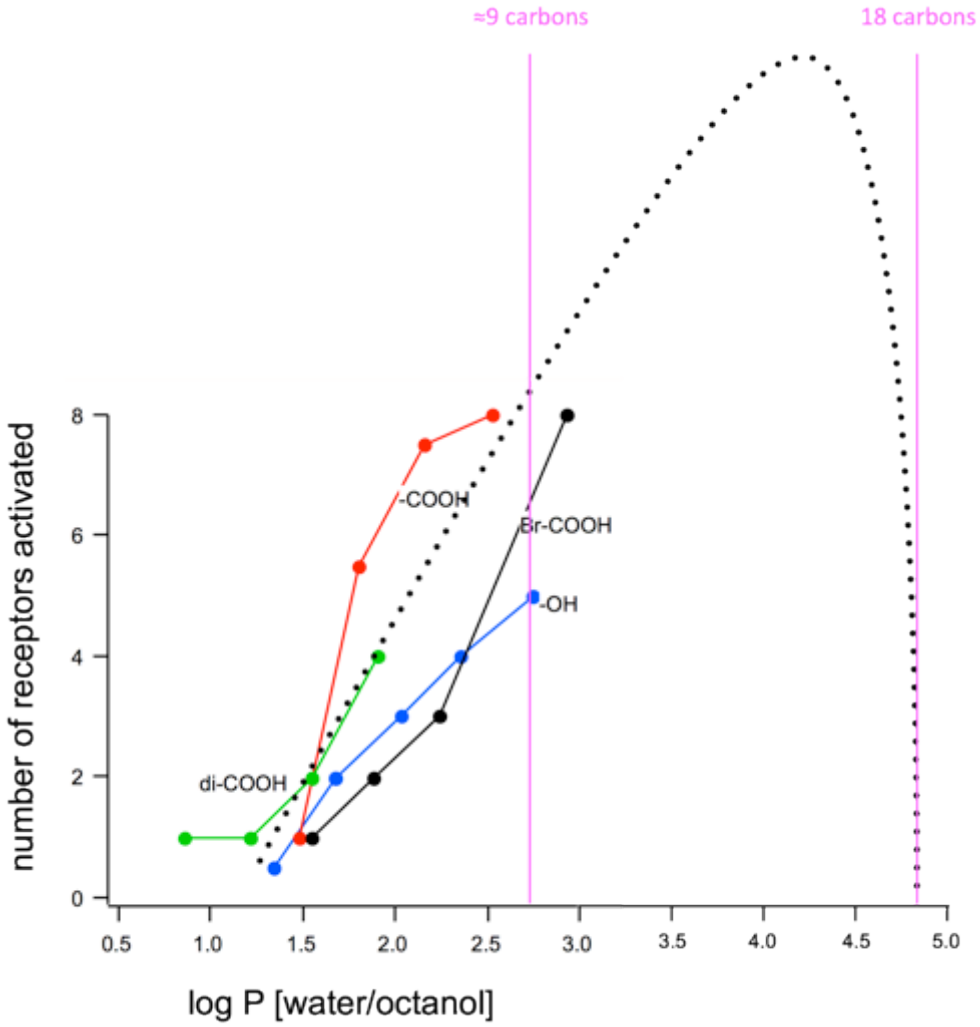



*Figure 39: A roughly linear relationship between size and number of receptors activated in smaller size molecules in the mouse. The upper limit of roughly 18 carbons implies a decrease in the number of recruited olfactory receptors at this size. Extrapolation of data from Nara et al 2011 (Nara et al. 2011). Courtesy L. Turin*

Figure 40 demonstrates there are, in the mouse, a relatively small number of narrowly responsive musk OSNs that respond to some musks but no other odorants. Again, this supports a relatively small number of specific musk odorant receptors, as humans have less than half the total number of olfactory receptors that mice do.

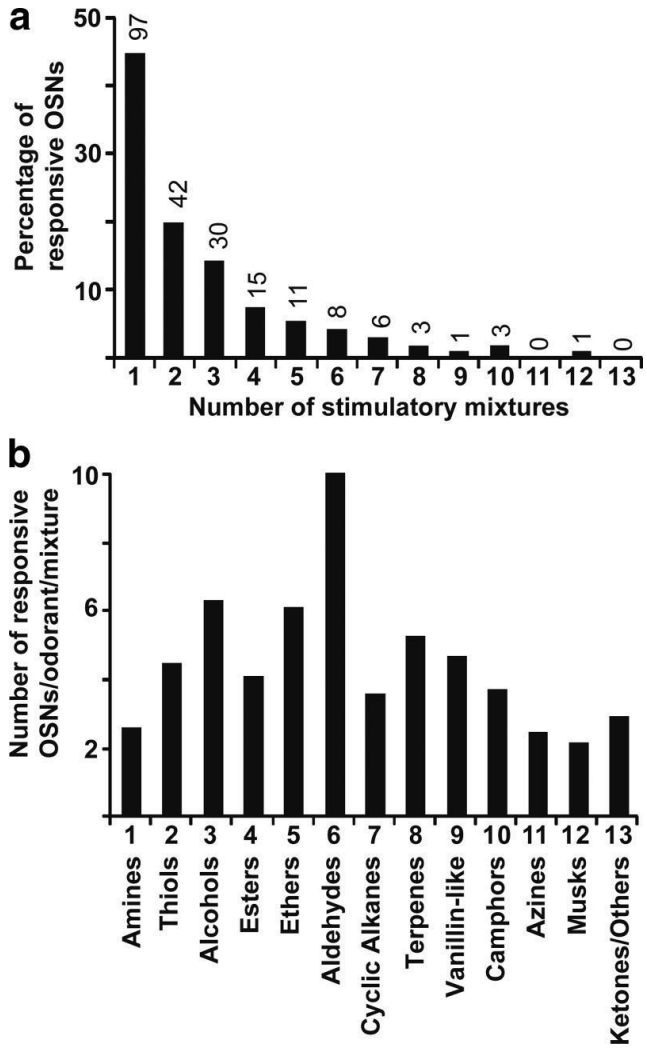



*Figure 40: Figure 4 from Nara et al (Nara et al. 2011) Quantitation of Mouse OSN responses to odorant mixtures. A: Individual OSNs responded to 1–12 mixtures, but most responded to only 1 or a few mixtures. The number of neurons that responded to the indicated number of mixtures is shown above each bar. B: Taking into account the number of odorants/mixture, the number of OSNs that responded to different mixtures varied, with the aldehyde mixture stimulating the most OSNs and the musk mixture stimulating the least.*

Interestingly, since these are live native OSNs being tested with a large number of odour mixtures and the musk mixture only stimulates a small number, which are not activated by any other tested odorants, this also indicates that a fair amount of the variation in their odour (if the mouse percepts of these molecules are comparable to the human) might come from variation in the activity of the receptor itself, and not from activation of

other receptors. Although the OSNs were not in olfactory mucosa and there were no OBPs present so stimulation of these receptors by by-products cannot be ruled out.

Unfortunately, we do not have the receptor identity of the OSNs with the demonstrated activities, so we do not know how many of these variants are due to receptor-level variations and how many to some other discrepancy. Although the data in Nara et al are difficult to interpret and given the small numbers of each OSN, caution must be exercised as to the significance of each finding, the implication is that the musk OSNs in mouse olfactory epithelium are small in number and narrowly tuned.

The mouse OR genome has been estimated to comprise over one and half thousand functional genes (Young, Friedman, Williams, Ross, Tonnes-Priddy & Trask 2002b) (Xinmin Zhang & Firestein 2002), whereas the functional human genome is below half of that (X Zhang & Firestein 2007). If there are perhaps only two to five mouse narrowly tuned musk receptors, we might expect that as the upper limit for humans too.

The First Identified Human Musk Olfactory Receptor.

Shirasu et al (Shirasu et al. 2014) were the first to identify a musk receptor in mice: Olfr1440, and humans: OR5AN1.

Using a combination of novel in-vivo imaging techniques with what they named OMP-spH mice (genetically engineered to co-express OMP, the olfactory marker protein unique to OSNs together with fluorescent exocytosis indicator synaptopHlourin), and c-Fos staining (to tag the areas with immediate-early gene expression, i.e. recently active tissues) they were able to demonstrate that mice exposed to muscone, ambrettone and cyclopentadecanone only activated a glomerulus within the antero-dorsomedial part of the olfactory bulb.

Expressing the glomerular receptor in the oocyte system and exposing to the same target olfactants demonstrated activation only with the same trio of macrocyclic musks, correlating well with the observed activity in the OB (Figure 41).

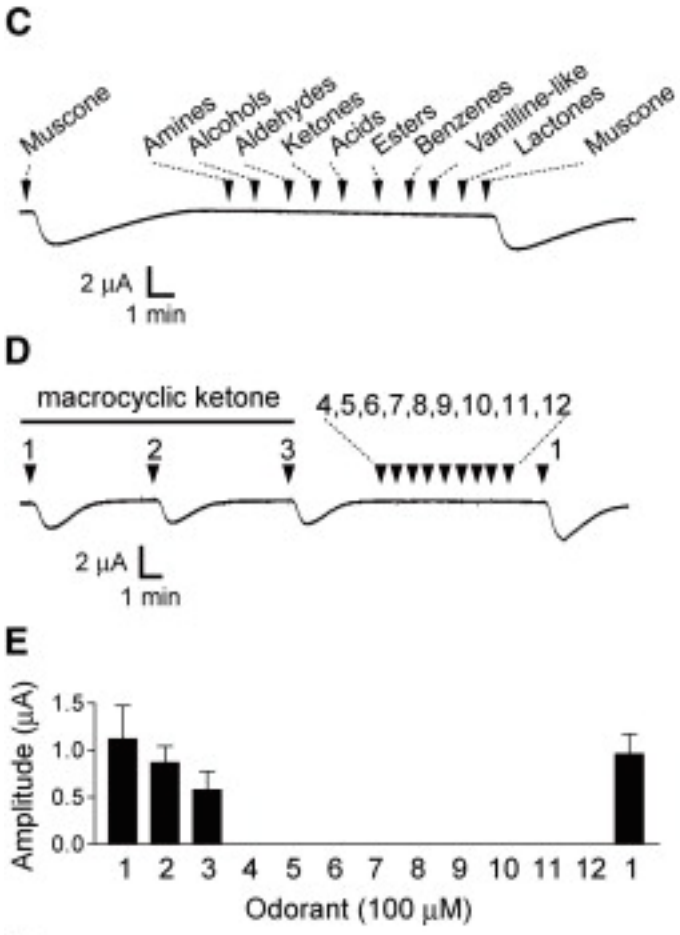



*Figure 41: Detail from Figure 7 from Shirasu et al (Shirasu et al. 2014), showing electrophysiological results for OR215-1 when exposed to musks and other odorants. "(C) Responsiveness of MOR215-1 to various odorant mixtures (each odourconcentration is 0.3 mM) in the Xenopus oocyte electrophysiology system. (D) MOR215-1 showed responses to macrocyclic ketone musks. 1, muscone; 2, cyclopentadecanone; 3, ambretone. Other musk odorants and analogs were 4, musk xylol; 5, musk ketone; 6, exaltolide; 7, ethylene brassylate; 8, tonalide; 9, Galaxolide; 10, cyclopentadecanol; 11, cyclopentadecane; 12, cyclohexanone. The concentration ofWhich detected each odorant was 100 mM. (E) Summary of response amplitude of MOR215-1-expressing oocytes for musk odorants and structural analogs in (D). Data represent mean values ± SE (n = 3). "*

Although most of the work was in mice they did identify the human homolog of the receptor: OR5AN1, with a 68% amino acid identity. In the HEK297-luciferase system they

demonstrated a stronger response to muscone than in the mouse but the next two closest in amino acid identity receptors, OR5A1 (64% similarity) and OR5A2 (62%) showed no response to muscone.

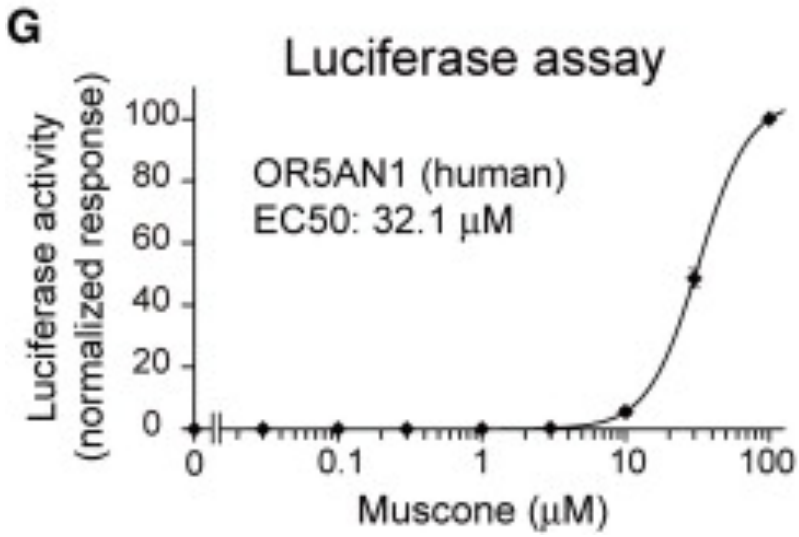



*Figure 42: Detail from Figure 7 in Shirasu et al [92]: luciferase assay for the human homolog olfactory receptor OR5AN1 exposed to muscone demonstating activation of the olfactory receptor in respnse to muscone exposure.*

The investigators did examine the chiral differences between the l- and d-enantiomers of muscone and demonstrated an approximately 100-fold difference in the EC50 between the two for OR215-1in the *Xenopus* expression system, which correlates with the human perceptions of their odour intensity (Kraft & Fráter 2001).

McClintock et al (McClintock et al. 2014), in a novel bioassay: the Kentucky *in vivo* odorant ligand-receptor assay, found five olfactory receptors which were enriched in the olfactory bulbs of a group of muscone-exposed animals. But when these ORs were expressed in a HANA3/luciferase heterologous system, only two of the five (Olfr1440 and Olfr235)

reacted to the muscone odorant. When tested against other musks, these two did not react to Galaxolide, Astrotone and Tonalid, any more than did the plasmid vector negative control.

The authors suggest membrane trafficking and other confounding factors as being the reason the three of the five suspected receptors did not react in the expression system. One possibility they do not consider is that the *in vivo* ORs are reacting to metabolite by-products of the musks rather than the musks themselves. Such metabolites are of course not present in the pure expression system.

Despite the slight inconsistencies, the findings imply again a small set of mouse ORs which react to musks, some of which are narrowly tuned in broad agreement with the findings of Nara et al.

This paper confirms OR5AN1 as the only human homologue of the enriched mouse musk receptors, sharing between 68% and 81% sequence identity with the five putative mouse receptors.

Musks Are a Good Probe Molecule

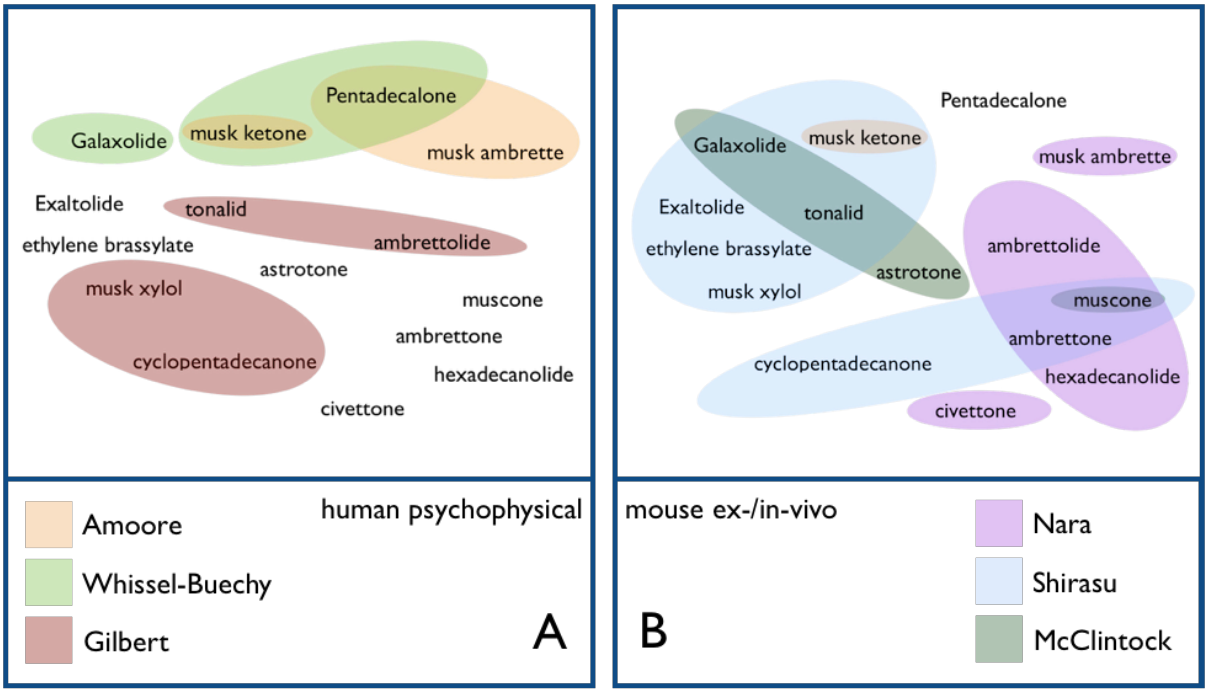



*Figure 43: Musks segregate into multiple groups in mice and humans. Musks are grouped by colour according to the reference. Spacial separation of groups of the same colour demonstrate discrimination between the molecules according to the reference. Diagram A is the grouping in humans using psychophysical testing, there is a clear grouping of musk xylol and cyclopentadecanone vs other musks. Diagram B collates data from mouse in/ex-vivo studies of olfactory receptor function. Data from: Amoore (Amoore et al. 1977) , Whissel-buechy (Whissell-Buechy 1973), Glibert (Gilbert & Kemp 1996), Nara (Nara et al. 2011), Shirasu (Shirasu et al. 2014) and McClintock (McClintock et al. 2014). Tabular version of data in Appendix E.*

Musks activate at least one receptor in humans (OR5AN1) and only a very small number of olfactory receptors in mice as seen in Figure 43. Using musks as a probe molecule would reduce the signal from many neurons, and many receptors, to just a few, so any receptor-level variation would become that more obvious in a psychophysical test. For these reasons, I elected to use a musk as one of the probe molecules.

**Metallocenes**

There are a few other possible odorants for psychophysical assessment which vary in vibrations but not surface topology and are therefore predicted to vary in odour by the vibrational hypothesis.

Turin and Yoshii (Turin & Yoshii 2003) report that ferrocene and nickelocene smell quite different (spicy-camphoraceous and oily-chemical respectively) but the only change between the two molecules is that they contain a different metal ion within their "sandwich" structure. These two metals do not change the shape of the molecule as they are buried with two interlocking ring structures see Figure 44, yet their vibrational profiles are quite different.

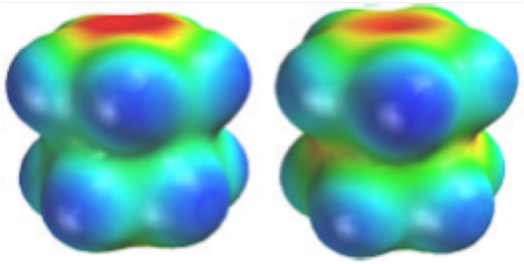


*Figure 44: From (Turin 2005) "Electron-density maps of ferrocene (left) and nickelocene with electrostatic potential mapped onto the surface. Structure, electron density and potential surfaces calculated by semi-empirical methods using Spartan with PM3 parameters. Red is more negative. There are small differences in ring spacing and charge distribution, whereas the odour of these two molecules is radically different: ferrocene smells spicy-camphoraceous, nickelocene smells oily- chemical."*

Unfortunately, these molecules are reported to be toxic and therefore not safe to use on human volunteers.

**Silicon Analogues**

In work from the early 90s Wannagat et al (Wannagat et al. 1993) examined linalool compounds where they replaced carbon with silicon, germanium, or tin. The derivatives were sometimes similar but often very different in odour character. Molecules containing tin instead of carbon tended to remain scentless, and both intensity and early character were changed with germanium substitution. The authors noted that this was difficult to explain by Amoore's stereochemical theory.

Doszczak et al (Doszczak et al. 2007) examined the sila-analogues of bourgenal and lilial, known ligands for the hOR17-40 receptor. As with many such papers the odour characters are merely stated as fact but they did notice a similar but different character

when some carbons replaced by silicon. They state that the difference in character disappears at the threshold level. Modelling the binding site, they found that the subtle alteration in the stereoelectronic properties predicted the alteration in threshold in both HEK293 and spermatozoa (which express the receptor hOR17-40) bio assays.

Since there are stereochemical reasons for the variation in odour, silicon analogues are not a good psychophysical probe odorant (leaving aside their difficulty in production).

All of these odorants have been reported to vary in odour when a simple shape model would not predict it. It is also important to examine molecules which may have a similar smell when the vibrational hypothesis would predict otherwise. The differing odours of some enantiomers have long been given as the classic counter, as they contain all the same molecules merely in a left or right-handed form. In the modern vibrational hypothesis, this is accounted for by the fact that the receptors themselves are chiral and therefore a different response is to be expected.

Instead of deuterated molecules, 13C isoptomers have been proposed as an ideal negative control molecular probes for the vibrational hypothesis. Although the carbon isotope will alter some physiochemical properties such as association/dissociation rates, they do not significantly alter the vibrational frequencies (Klika 2013).

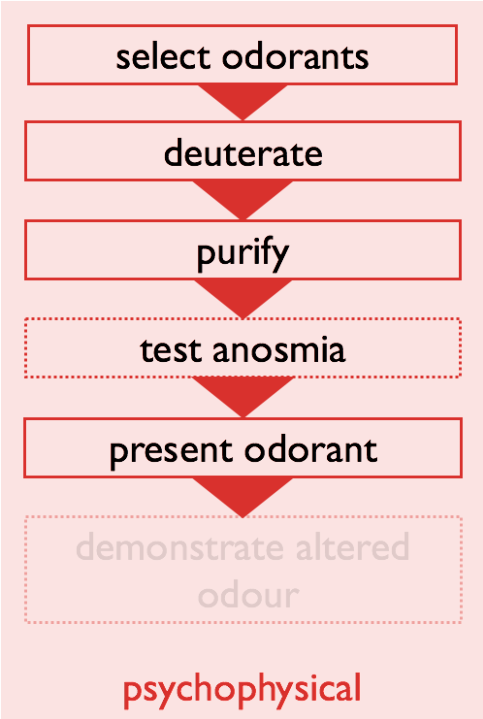



*Figure 45: Pathway for psychophysical experiments*

## Psychophysical experiment: Toluene Pilot Study

As a test of the research method and viability of the psychophysical technique, I conducted a pilot study comparing deuterated toluene to its hydrogenated version with three volunteers.

### Design

A 2-element forced-choice comparison of unpurified deuterated and non-deuterated toluene naïve smellers.

### Setting

Laboratory experiment with volunteers

**Participants**

The three testers were fellow postgraduate students at the London Centre for Nanotechnology, as this was a pilot study, they were not assessed for olfactory ability or nasal disease.

**Outcome Measures**

The outcomes were the successful identification of a same/different relation between successive randomised samples of deuterated and hydrogenated laboratory-grade toluene.

**Conduct of Experiment**

Toluene and deuterated Toluene were obtained from Sigma-Aldrich and were not GC purified. There were only two samples, one of each type. The sample containers were visibly marked and known to the experimenter.

The samples were presented in an AxxB format, with an identified sample (A or B) presented first, then two unidentified samples (x). For the identified sample; "A" or "B", the sample was presented to the volunteer and verbally identified. For the unidentified sample; "x" the sample was randomly selected from the bag and offered to the subject, with no verbal interaction.

This format always allowed a comparison between a known and unknown odour, as well as a comparison between the unknown odours. Subjects were asked to identify the unknown odour samples by reference to the known.

Opaque eye protection glasses blinded the sniffers. The inter-sniff time was not controlled and varied from 15s to 2 minutes.

Toluene is not a pleasant odour to smell repeatedly, as discussed, and there were only 16 attempts at identification for each volunteer.

**Statistical Analysis**

The outcomes of the experiments were recorded by hand. They were entered in R and the exact binomial significance calculated as described below, using the Pearson-Clopper method.

These experiments are classical examples of a binomial experiment: they have a fixed number of independent trials with only two possible outcomes of fixed probability. Since they have a low number of trials the exact probability of the result can be calculated and compared to chance.

## Psychophysical Experiment: Acetophenone

Acetophenone (see odorant selection section in methods) is a pleasant-smelling small molecule, easily available in relatively pure deuterated form (Sigma-Aldritch). As such it fulfilled many of the criteria for a good probe molecule as discussed above.

Importantly, because it was a pleasant-smelling chemical, the volunteers would tolerate multiple sniffs of the molecules, allowing a high statistical power. For these experiments, I made a great many ultra-high purity samples via preparative gas chromatography and recruited trained and untrained smellers to attempt to demonstrate a difference in odour character between deuterated and non-deuterated odorants.

### Design

A 2-element forced-choice comparison of randomised unpurified deuterated and non-deuterated acetophenone in naïve and trained smellers.

### Setting

The trials were conducted in the laboratory, at home or at the perfumer's place of work. There were no strong environmental odours, but there was no attempt to control for any background smell as this was unlikely to influence the outcome as it was constant between samples.

### Participants

Six subjects were recruited: three professional perfumers and three untrained. As discussed, professional smellers do have a slightly better discriminative ability in comparing and identifying odours. To maximise the chances of finding a difference detectable by the human olfactory sense I recruited perfumers and untrained volunteers.

Both naive and professional smellers were contacted by personal invitation or through the BSP (British Society of Perfumers) mailing list. All were invited to participate with an information sheet and a verbal discussion of the aims of the project. All volunteers were aware of the vibrational hypothesis to a greater or lesser degree.

All volunteers were given the information sheet to read before the tests and signed an informed consent. All but one (MS) completed a SNOT-22 and UPSIT smell test. He had no reported nasal symptoms. (Because of this the results for MS were excluded from publication, but are presented in the results here for completeness.)

Participants were included if they were able to provide informed consent. The only exclusion criteria were general anosmia defined as an UPSIT score less than 25 (no exclusions), nasal disease: either self-reported or defined as a SNOT-22 greater than 7 (Gillett et al. 2009).

Assessment of Olfactory Ability

In these experiments, unlike many others in clinical olfaction testing, a near perfect sense of smell is desirable. The aim is not to elucidate the underlying defect in the volunteer's sensorium, but to examine the sensorium itself. To do this I used two methods to attempt to exclude hyposmia or other abnormalities of olfaction. All subjects were excluded if their olfactory abilities were significantly impaired. The first is to use a common, well-validated quality of life questionnaire, the 22-item Sino-nasal Outcome Test (SNOT-22) to exclude possible sinonasal disease, which would impact on olfactory function.

**Sino-Nasal Outcome Test-22 Questionnaire**

**Name:** [redacted] **Date:** 15th JUNE 2010

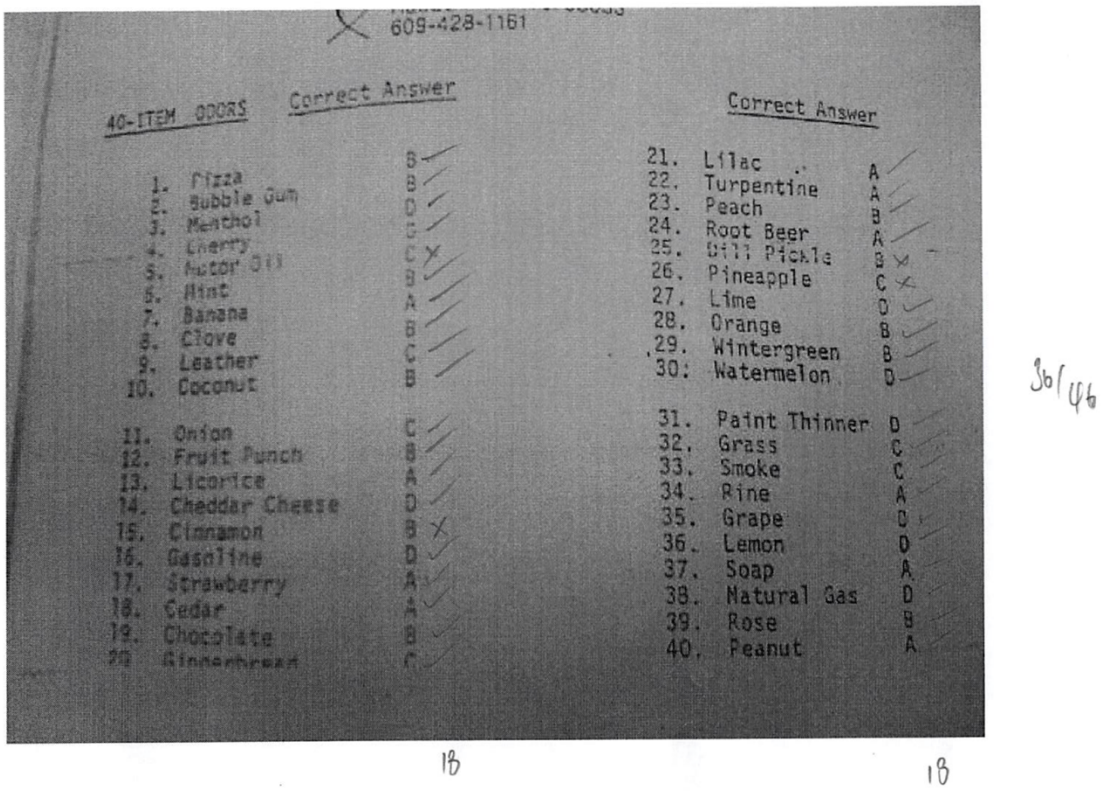
Below you will find a list of symptoms and social/emotional consequences of your nasal disorder. We would like to know more about these problems and would appreciate you answering the following question to the best of your ability. There are no right or wrong answers, and only you can provide us with this information. Please rate your problems, as they have been over the past two weeks. Thank you for your participation.

| **A:** Considering how severe the problem is when you experience it and how frequently it happens, please rate each item below on how 'bad' it is by circling the number that corresponds with how you feel using this scale → | **No problem** | **Very mild problem** | **Mild or slight problem** | **Moderate problem** | **Severe problem** | **Problem as bad as it can be** | |
|---|---|---|---|---|---|---|---|
| 1. Need to blow nose | 0 | 1 | (2) | 3 | 4 | 5 | |
| 2. Sneezing | 0 | 1 | (2) | 3 | 4 | 5 | |
| 3. Runny nose | 0 | 1 | (2) | 3 | 4 | 5 | |
| 4. Cough | 0 | (1) | 2 | 3 | 4 | 5 | |
| 5. Post nasal discharge (dripping at the back of your nose) | 0 | (1) | 2 | 3 | 4 | 5 | |
| 6. Thick nasal discharge | (0) | 1 | 2 | 3 | 4 | 5 | |
| 7. Ear fullness | (0) | 1 | 2 | 3 | 4 | 5 | |
| 8. Dizziness | (0) | 1 | 2 | 3 | 4 | 5 | |
| 9. Ear pain | (0) | 1 | 2 | 3 | 4 | 5 | |
| 10. Facial pain/pressure | (0) | 1 | 2 | 3 | 4 | 5 | |
| 11. Difficulty falling asleep | 0 | (1) | 2 | 3 | 4 | 5 | |
| 12. Waking up at night | 0 | 1 | (2) | 3 | 4 | 5 | |
| 13. Lack of a good night's sleep | 0 | 1 | (2) | 3 | 4 | 5 | |
| 14. Waking up tired | 0 | 1 | (2) | 3 | 4 | 5 | |
| 15. Fatigue | 0 | (1) | 2 | 3 | 4 | 5 | |
| 16. Reduced productivity | 0 | (1) | 2 | 3 | 4 | 5 | |
| 17. Reduced concentration | 0 | (1) | 2 | 3 | 4 | 5 | |
| 18. Frustrated/restless/ irritable | 0 | (1) | 2 | 3 | 4 | 5 | |
| 19. Sad | (0) | 1 | 2 | 3 | 4 | 5 | |
| 20. Embarrassed | (0) | 1 | 2 | 3 | 4 | 5 | |
| 21. Sense of taste/smell | (0) | 1 | 2 | 3 | 4 | 5 | |
| 22. Blockage/congestion of nose | (0) | 1 | 2 | 3 | 4 | 5 | |
| **TOTAL:** | | 7 | 6 | ___ | ___ | ___ | |
| | | 19 | | | | **GRAND TOTAL:** | (19) |



*Figure 46: Example of completed SNOT-22 questionnaire for a volunteer*

The SNOT-22 is a patient-reported outcome measure (PROM) designed for the assessment of chronic Rhinosinusitis with or without nasal polyposis. It is a revision of the older SNOT-20 with the addition of two items asking about nasal obstruction and olfaction (Browne et al. 2007). It is mainly used as a measure of the outcomes of various treatments for nasal disorders and has recently been validated and shown to be reliable and internally consistent. (C Hopkins et al. 2009)


609-428-1161

| 40-ITEM ODORS | Correct Answer | | Correct Answer |
|---|---|---|---|
| 1. Pizza | B | 21. Lilac | A |
| 2. Bubble Gum | B | 22. Turpentine | A |
| 3. Menthol | D | 23. Peach | B |
| 4. Cherry | C | 24. Root Beer | A |
| 5. Motor Oil | C | 25. Dill Pickle | B |
| 6. Mint | B | 26. Pineapple | C |
| 7. Banana | A | 27. Lime | D |
| 8. Clove | B | 28. Orange | B |
| 9. Leather | C | 29. Wintergreen | B |
| 10. Coconut | B | 30. Watermelon | D |
| 11. Onion | C | 31. Paint Thinner | D |
| 12. Fruit Punch | B | 32. Grass | C |
| 13. Licorice | A | 33. Smoke | C |
| 14. Cheddar Cheese | D | 34. Rine | A |
| 15. Cinnamon | B | 35. Grape | D |
| 16. Gasoline | D | 36. Lemon | D |
| 17. Strawberry | A | 37. Soap | A |
| 18. Cedar | A | 38. Natural Gas | D |
| 19. Chocolate | B | 39. Rose | B |
| 20. Gingerbread | C | 40. Peanut | A |

18 18

*Figure 47: UPSIT score sheet for volunteer*

The second pre-test selection criterion for the experiment is a normosmic score on the University of Pennsylvania's Smell Identification Test (UPSIT). Developed by Dr. Richard Doty and others at U Penn and first described in 1984 (Doty et al. 1984), the UPSIT is a standardized, microencapsulated "scratch and sniff" booklet of forty Microfragrance (Tm) (3M company, Minneapolis, MN, USA) samples. Each sample is presented at super-threshold level, detection thresholds are not examined. The test can be self-administered, each odour

is smelt and then a forced-choice identification is made from 4 options, the full test takes about 10-15 minutes.

The UPSIT has been shown to have good test-retest reliability and correlates well with odour threshold detection measurements. It is sensitive and has been shown to detect olfactory dysfunction in a wide variety of disease conditions.

Several other methods for the measurement of nasal function and olfactory ability are possible, but were not used for various reasons. For instance: simply asking participants about their olfactory and nasal function has been shown to be a very poor predictor of actual olfactory ability (Landis et al. 2003),(Mackay-Sim et al. 2006), nasendoscopic inspection of the nose and olfactory cleft would require topical anaesthesia, which alters olfactory function (Welge-Lüssen et al. 2004), and the reduced 3-item Q-SIT is not very sensitive for mild to moderate hyposmics (Jackman & Doty 2005).

**Outcome Measures**

The outcomes were the proportion of successful identification of a same/different relation between successive randomised samples of deuterated and hydrogenated highly-purified acetophenone for each volunteer, a binomial experiment.

**Statistical Analysis**

The outcomes of the experiments were recorded by hand. They were entered in R and the exact binomial significance calculated as described below, using the Pearson-Clopper method.

These experiments are examples of a binomial experiment: they have a fixed number of independent trials with only two possible outcomes of fixed probability. Since they have a low number of trials the exact probability of the result can be calculated and compared to chance.

The point of these experiments was not to demonstrate that most testers can detect a difference between he samples, but that at least one can detect a difference that the standard model of olfaction cannot account for.

There were several subgroups within the population, which I examined further, although they were underpowered. I looked for a possible training set effect, i.e. that identifying the molecules beforehand: "this is A, this is B", might improve the chances of making the identification. I also looked at whether the perfumery professionals were better at making the identification.

**Sample Preparation**

Having selected acetophenone as my first probe molecule, both hydrogenated and deuterated acetophenone [d-8] samples were obtained from Sigma-Aldrich and CDN isotopes. The concentration of hydrogenated ACP was 99% and the deuterated form was 97% with 99% deuteration.

I used preparative gas chromatography (step three in Figure 48), the standard in the fragrance industry for the purification and identification of odorant samples, in an attempt to control the means by which one odorant can be distinguished from another. The sensitivity of the human olfactory system is such that it is said to be able to distinguish the presence of 30-40 molecules of a substance, so the samples needed to be so pure that the likelihood of another substance present in one test sample and not the other did not approach that cut-off.

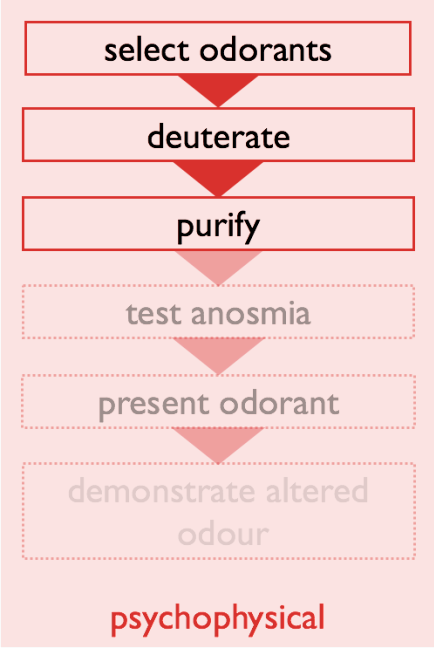



*Figure 48: Aims and outline of psychophysics strand: purification step*

The assessment of molecular odour character is performed with a gas chromatograph modified with a 'smelling port'. The odour character of the peaks exiting the column can then be assessed by the experimenter smelling them coincident with the detector peak. Comparison of smell characters between molecules is difficult in this situation because each character has to be analysed and remembered for several minutes before comparing with the next molecule transiting the GC. This technique is therefore limited to subjects with extensive olfactory practice.

The technique I used for the preparation of all samples required for the psychophysical experiments described here is the same as that described by Dr Christina Zelano from UC Berkeley (pers comm) for the capture of odorants from the smelling port into a container such as a capped polyethylene Eppendorf tube. For most molecules the odour is retained within the tube for long enough that it can be reopened multiple times which allows multiple assessments of early character and facilitates swift comparison between two molecules.

**Sample Purification**

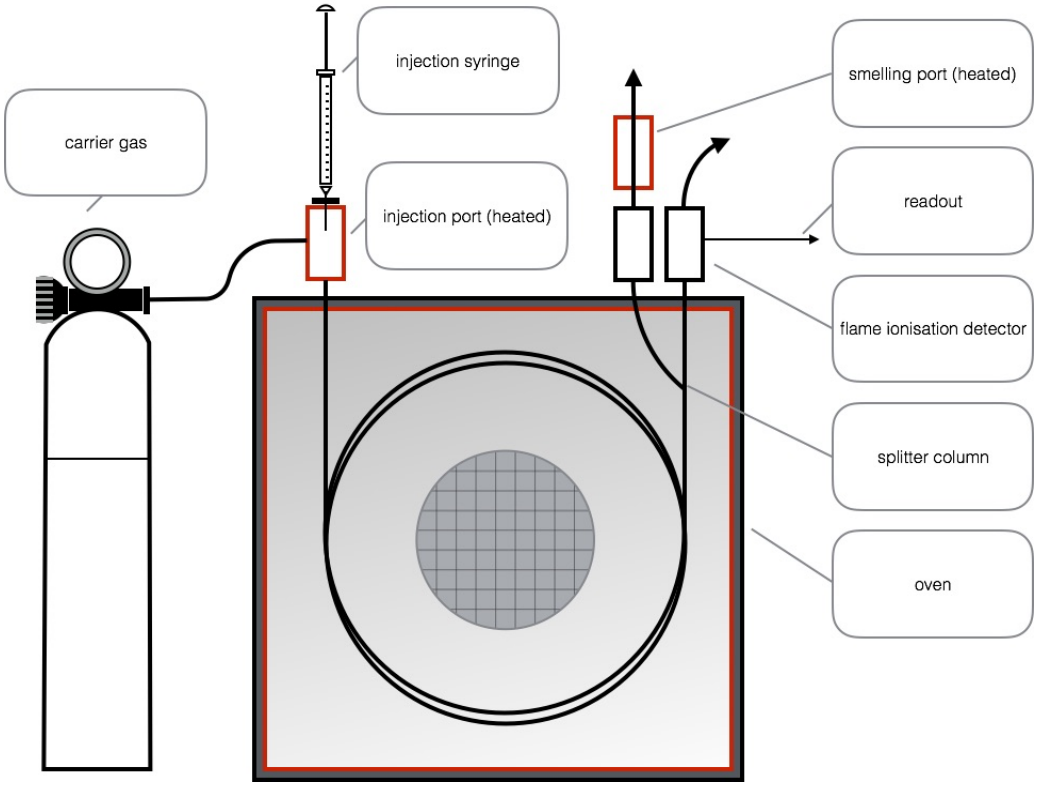



*Figure 49: Preparative gas chromatography diagram of sniff-GC*

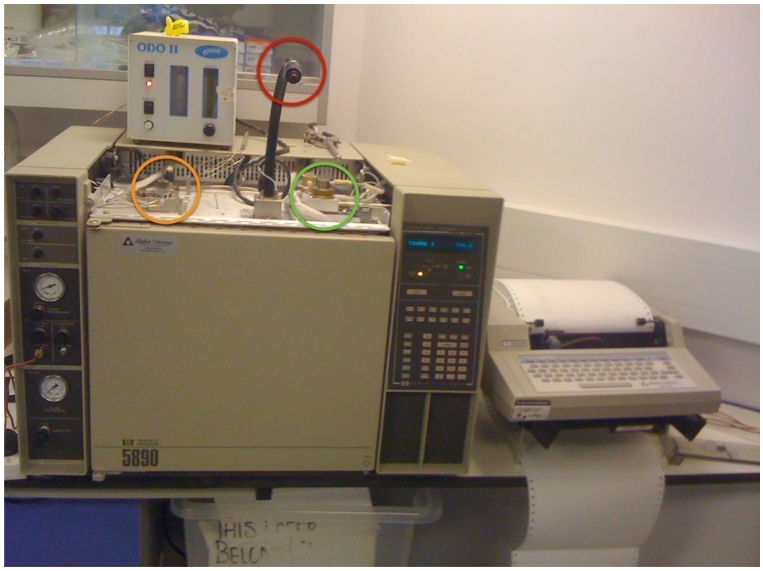



*Figure 50: Preparative gas chromatography set up. The injection port can be seen within the orange circle. Red identifies the smelling port and green, the flame ionisation detector.*

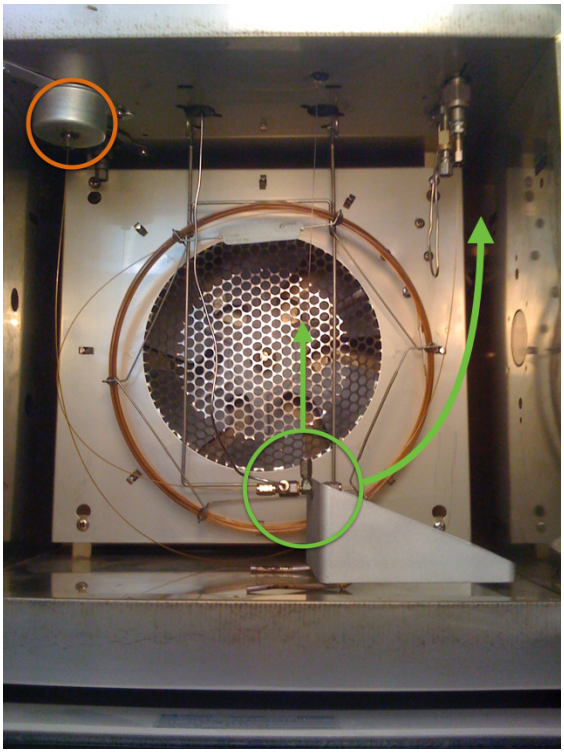
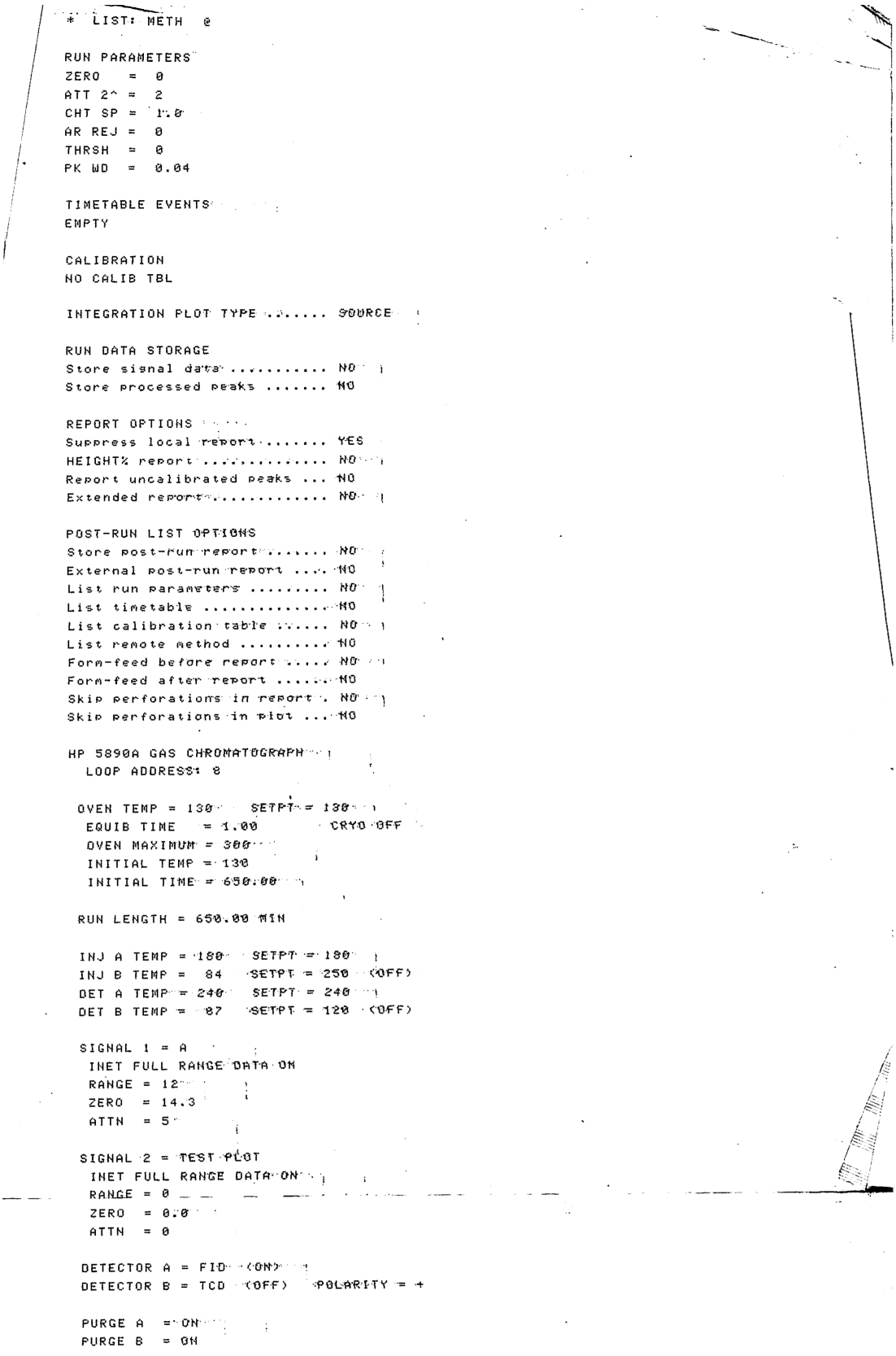


*Figure 51: View of the GC oven. Orange is the line from the injection port and green identifies the splitter adapter*

The sample preparation method is standard for all the tested odorants: in a HP 5830 gas chromatograph (or GC) with flame ionisation detector, using Helium as makeup gas with a 15m column and splitter adaptor with a "smelling port" as in Figures 49, 50 and 51. The gas settings are given in Table 3 and the full settings report in Figure 52.

| GAS | SETTING |
|---|---|
| **H2** | 28psi |
| **N2** | 4.0 bar |
| **AIR** | 18 psi |
| **HE** | 50 psi |
| **N2 MAKEUP** | off |

*Table 3: Gas settings for gas chromatograph.*



```
* LIST: METH @

RUN PARAMETERS
ZERO   =  0
ATT 2^ =  2
CHT SP =  1.0
AR REJ =  0
THRSH  =  0
PK WD  =  0.04

TIMETABLE EVENTS
EMPTY

CALIBRATION
NO CALIB TBL

INTEGRATION PLOT TYPE ..... SOURCE

RUN DATA STORAGE
Store signal data ........... NO
Store processed peaks ....... NO

REPORT OPTIONS
Suppress local report ....... YES
HEIGHT% report .............. NO
Report uncalibrated peaks ... NO
Extended report ............. NO

POST-RUN LIST OPTIONS
Store post-run report ....... NO
External post-run report .... NO
List run parameters ......... NO
List timetable .............. NO
List calibration table ...... NO
List remote method .......... NO
Form-feed before report ..... NO
Form-feed after report ...... NO
Skip perforations in report . NO
Skip perforations in plot ... NO

HP 5890A GAS CHROMATOGRAPH
  LOOP ADDRESS: 8

 OVEN TEMP = 130   SETPT = 130
  EQUIB TIME   = 1.00      CRYO OFF
  OVEN MAXIMUM = 300
  INITIAL TEMP = 130
  INITIAL TIME = 650.00

 RUN LENGTH = 650.00 MIN

 INJ A TEMP = 180   SETPT = 180
 INJ B TEMP =  84   SETPT = 250  (OFF)
 DET A TEMP = 240   SETPT = 240
 DET B TEMP =  87   SETPT = 120  (OFF)

 SIGNAL 1 = A
  INET FULL RANGE DATA ON
  RANGE = 12
  ZERO  = 14.3
  ATTN  = 5

 SIGNAL 2 = TEST PLOT
  INET FULL RANGE DATA ON
  RANGE = 0
  ZERO  = 0.0
  ATTN  = 0

 DETECTOR A = FID  (ON)
 DETECTOR B = TCD  (OFF)   POLARITY = +

 PURGE A  = ON
 PURGE B  = ON
```

*Figure 52: Full GC methods printout*

A 5 −10µL aliquot of the substance was injected into the inlet. Only distilled odorants of 97% purity or higher were used to reduce the likelihood of contaminants with equal transit times through the column. The sample peak was detected at the FID and captured at the port with a several millisecond lag between detection and the substance being detectable by the operator at the port. The Eppendorf was placed over the sniffing port for the duration of the peak at the FID to capture the sample and removed as soon as the peak passed as shown in Figure 53. The resolution and time were set so that there were no coincident peaks within 15s of the sample peak. The estimated amount of the sample was 1 microgram per capped tube. The transit time was usually about 2.5 min.

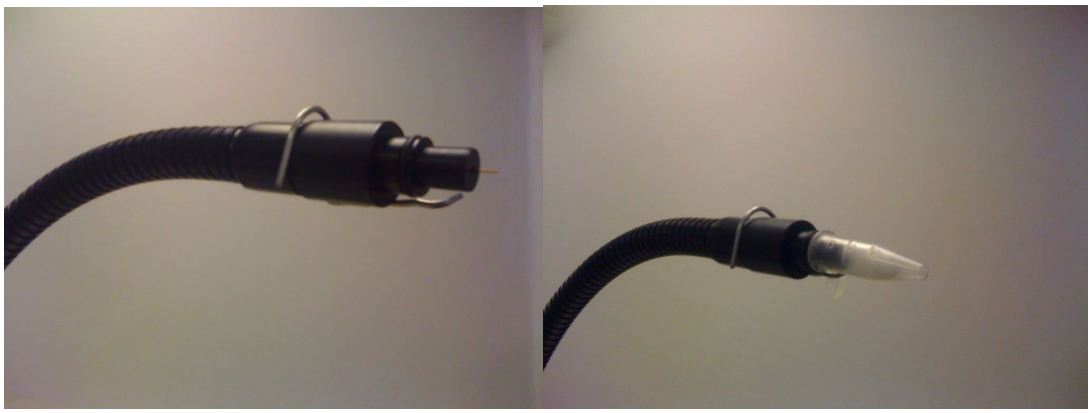


*Figure 53: GC sniffing port tip with clip modification to hold Eppendorf.*

**Preparative Gas Chromatography Method**

The gas chromatograph settings were: oven at constant temperature of 130°C (no ramp), FID temp at 180°C with a 15m packed column connected as shown in Figure 51. Samples were injected approximately every five minutes with the make-up gas and settings run continuously. The estimated amount of the sample captured is about 1 microgram per capped tube. Multiple passes could be captured into the same tube if required but this was unnecessary in the case of acetophenone.

In the case of acetophenone, purified samples were collected from the smelling port during the peak shown in Figure 53 into the Standard capped Eppendorf LDPE tubes, prepared by packing with approximately 1 $cm^3$ of sterile cotton wool and chilled to -4° C. All samples were prepared in this way to maximise purity and reduce the likelihood of identifying A/B by cues other than the odour of the odorant under investigation. All contact was with clean latex clinical gloves and acetone cleaned and air-dried instruments to avoid odour contamination.

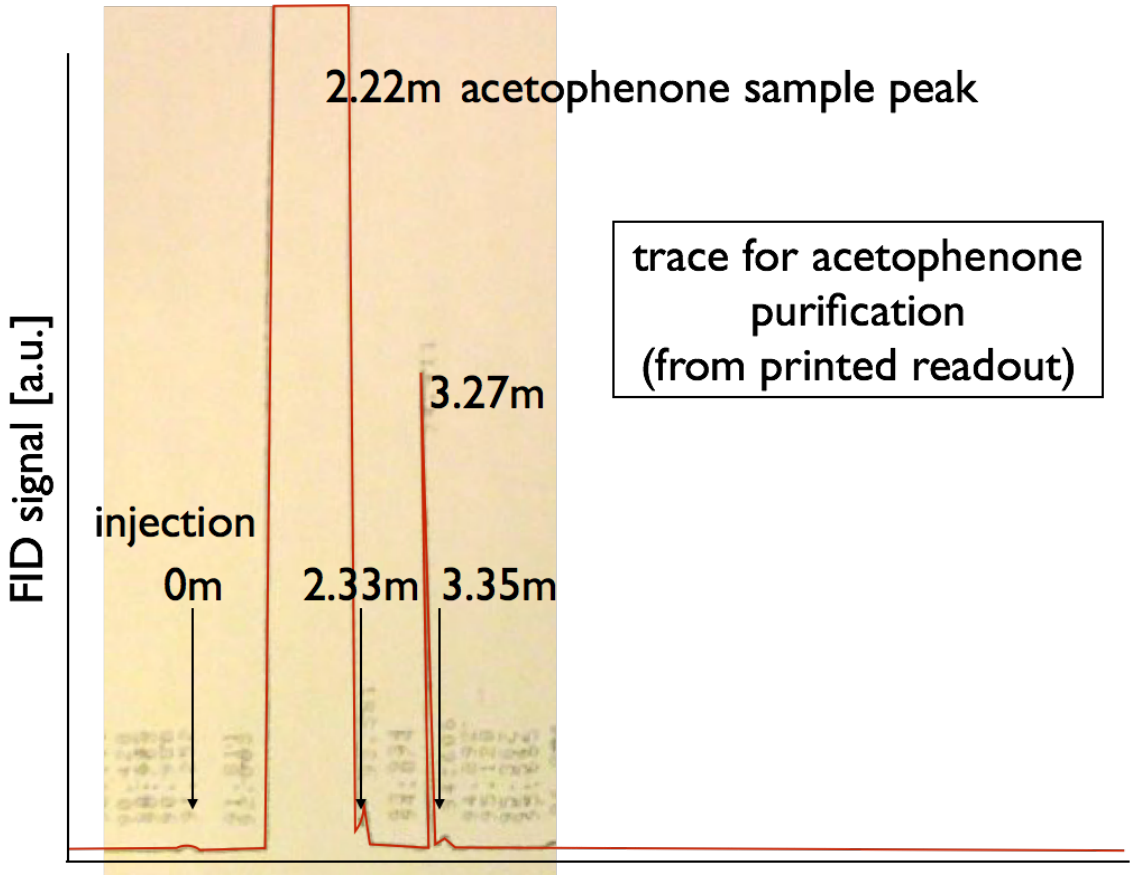



*Figure 54: GC trace for acetophenone with overlay*

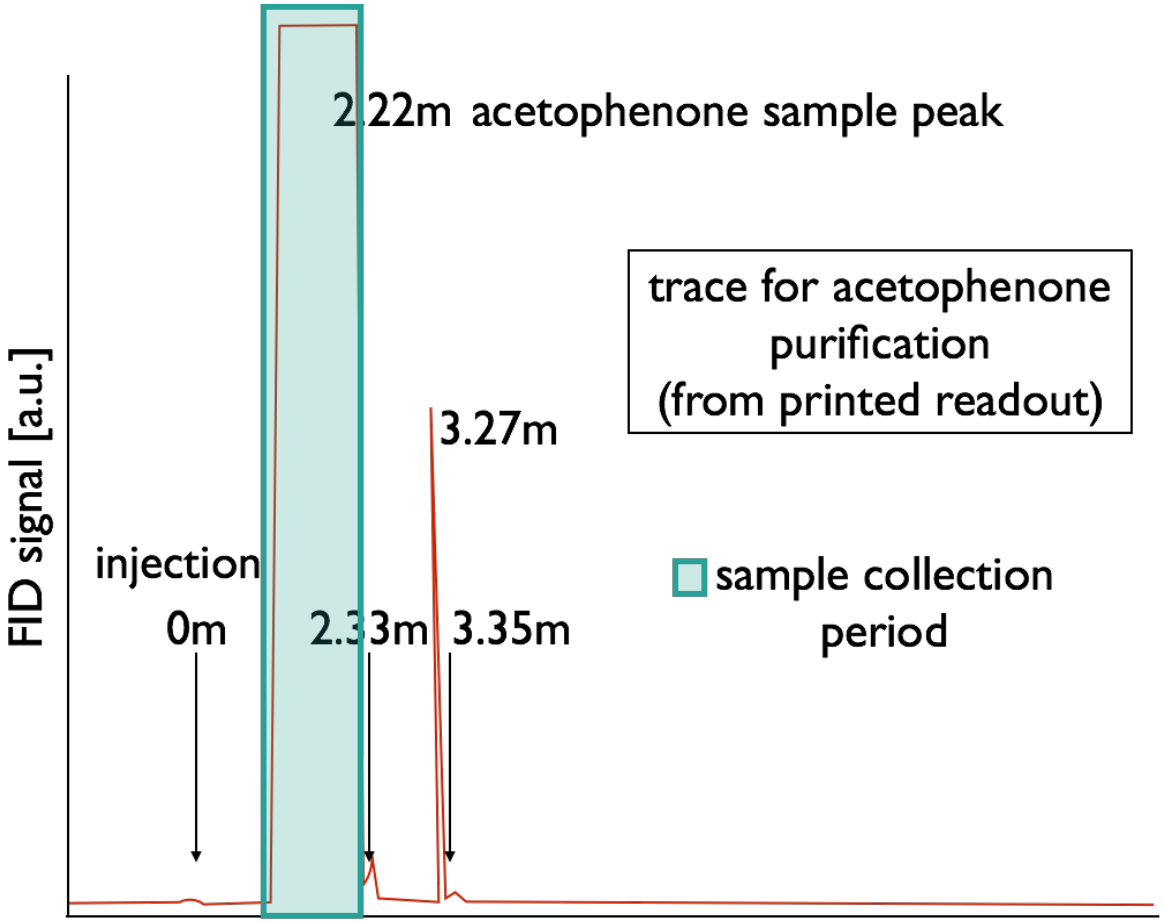



*Figure 55: Sample Collection Period overlaid on trace from Figure 54*

**Reinjection Trace and Overlay**

To ensure that the purification method remained robust every 10th to 15th sample was collected and reinjected. The traces remained virtually identical, although volumes were obviously significantly decreased. Unfortunately, the collected sample amounts were too small to check via NMR or IR spectroscopy and mass spectroscopy was too expensive.

**Sample Presentation**

Although Dr Zelano's method originally described using a bare Eppendorf tube, both odorants required modification of this method to maximise the longevity of their odour within tube.

For each subject, a total of at least twenty samples, ten of each of the investigated pair were produced in batches of five samples of an odorant at a time. They were stored in

the freezer at -4°C until ready and used within a day of preparation. (The intensity of the odour rapidly diminished after this.)

To double blind the experimenter and volunteer, each Eppendorf was labelled with UV-visible ink. Each sample was assigned a unique identifier number for that trial, marked on the cap of the Eppendorf tube. The number was not visible in normal light, but under a portable UV light source (Helix) the sample number could be identified. The samples were thus indistinguishable to both experimenter and volunteer until after the volunteer had smelt and handed them back to the experimenter. The number could then be recorded and the nature of the sample known.

**Randomisation**

The samples were mixed together in a cardboard box that was thoroughly shaken before every sample was blindly selected. The sample was smelt, identified and replaced before the box was shaken again. This requirement of random selection *with replacement* places the chance of any pairwise comparison being the same or different at 50%. Each subject sniffed ten pairs of eppendorfs followed by short rest to attempt to control any habituation. Multiple sets of 10 pairs per set were examined as often as could be tolerated. Two subjects (TG and DR) were examined in one day, but the other three were examined over multiple days with several different sets of odorant pairs.

The nature of each pair was recorded (e.g. AA, AB, BB) and the volunteer asked - did they smell the same or different? (A yes/no forced choice test). Some volunteers did attempt to identify the samples but this was discouraged. Some volunteers were given a training set prior to the blinded tests (DR, TS, MS).

Because the samples were all re-randomised with every trial and double-blinded, I sat with the subject during the procedure. It was not possible to smell the samples from even a short distance away.

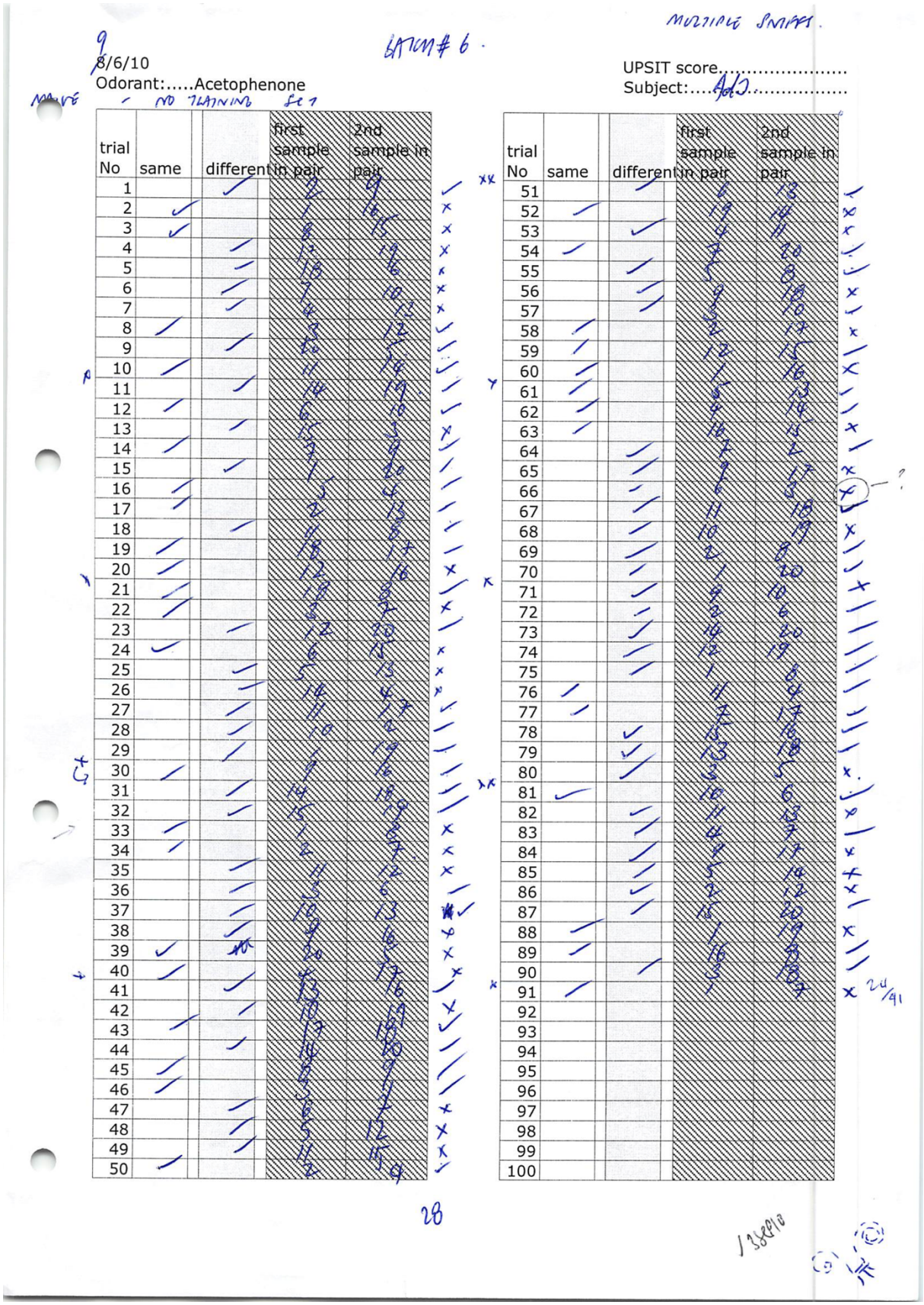



*Figure 56: Sample result recording sheet for acetophenone*

**Statistical analysis**

Data were entered into Microsoft Excel 2011 (Microsoft Corporation). The data in spreadsheet form were exported as .csv files and imported into R studio.

In R Studio the experimental data were summarised by a function I wrote called "binomilise" into a summary data frame including the binomial P value and confidence intervals. The proportions of each group were checked using the prop.test function as a two-sided test Chi-squared test for equality of proportions.

I compared subgroups by two-sample two-tailed t-tests, as well as examining the impact of individual sample order with a Tukey HSV comparison.

## Psychophysical Experiment: Musks

### Design

A 2-element forced-choice comparison of unpurified deuterated and non-deuterated Exaltone in naïve smellers.

### Setting

The experiments took place within a laboratory space at the Ear Institute. There were no strong environmental odours, but there was no attempt to control for any background smell as it was constant between samples and therefore unlikely to influence the outcome

### Participants

For most experiments, untrained volunteers demonstrated such a significant ability to detect a difference in the odour character that trained smellers were not needed. Volunteers were recruited from co-workers in the Ear Institute.

All were invited to participate with an information sheet and a verbal discussion of the aims of the project. All volunteers were given the information sheet to read before the tests and signed an informed consent.

### Exclusion Criteria

As previously, participants were included if they were able to provide informed consent. The only exclusion criteria were general anosmia defined as an UPSIT score less than 25 (no exclusions), a demonstrated specific anosmia to musks (1 excluded) or nasal disease, either self-reported or defined as a SNOT-22 greater than 7 (Gillett et al. 2009).

In addition to the general exclusion criteria, all subjects were checked to see whether they had a STV to musks.

Failure to detect an odour in either of two presented samples excluded them from any further trials (only one subject was excluded in this way). If successful the subject was

presented with a deuterated sample and asked if they could detect a "burnt" or "nutty" smell. Several other samples were presented until the subject was sure they could tell the difference between the two sample types.

**Outcome Measures**

The outcomes were the successful identification of a same/different relation between successive randomised samples of deuterated and hydrogenated highly purified Exaltone.

**Conduct of Experiment**

I selected the following molecules based on their availability, tolerance and safety, as discussed previously. I chose Cyclopentadecanone (Exaltone®) as the musk "probe molecule" for its stability and strong odour. As with the other musks, when it was first deuterated there was an obvious alteration in its odour quality. This alteration was preserved on gas chromatography purification, indicating there was a significant change in the odour which was almost certainly a result of the chemical change.

All synthetic chemistry, including the partial deuteration of the musks and assessment of same was undertaken at Vioryl SA, a fragrance company in Athens, by my co-workers there.

The molecule was deuterated by a catalytic method and although this does not give a 100% deuteration yield, it does use mild conditions (about 160°C and 5 atm) which minimises side products and potential contaminants. Unfortunately, this method also excludes any molecules with double bonds as it is a hydrogenation reaction and thus limits the choices for other possible musks. Collaborators at the Alexander Fleming Institute in Athens and Vioryl SA, a commercial perfumery company prepared a selection of the commercially available musks and were successful with three other molecules: the

macrocycles cyclopentadecanolide (Exaltolide®), 1,4 dioa cyclohepta-decane-5,17-dione (Astrotone ®) and, because the double bonds in an aromatic ring are not affected, the polycyclic 1-(3,5,5,6,8,8,-heaxmethyl-1,6,7-dihydronaphthalen-2-yl) ethanone (Tonalid ®).

**Method for the Deuteration of Masks**

628 mg of commercially available Cyclopentadecanone (2.8 mmol) was diluted in 500 µL of cyclohexane and mixed with 12 ml of $D_2O$ and 5 wt% Rhodium on Carbon (Rh/C) (126mg, 20 wt% substrate). All reagents were obtained from Aldrich.

In a Q-Tube™ (QLabtech, Tamarack, CT) pressure vessel at 160° C and 50 psi, under $H_2$ atmosphere the mixture was stirred for 48 hours. This mixture was then extracted with $Et_2O$ and filtered to remove the catalyst through a membrane filter. The filtrate was washed with brine, dried over $MgSO_4$ and concentrated in vacuo.

Silica gel chromatography was used to purify the residue, which resulted in 370 mg of product. 1H NMR spectroscopy was used to confirm Deuteration with p-anisic acid as the internal standard. The spectra of deuterated cyclopentadecanone in $CDCl_3$ is shown in Figure 57. Results of the spectroscopy were: in $CDCl_3$, (p-anisic acid 0.5 equivalents as an internal standard) δ 2.37 (m,0.2H), 1.59 (m,0.22H), 1.30-1.20 (m,1.72H) 0.84–0.87 (m,0.25H).

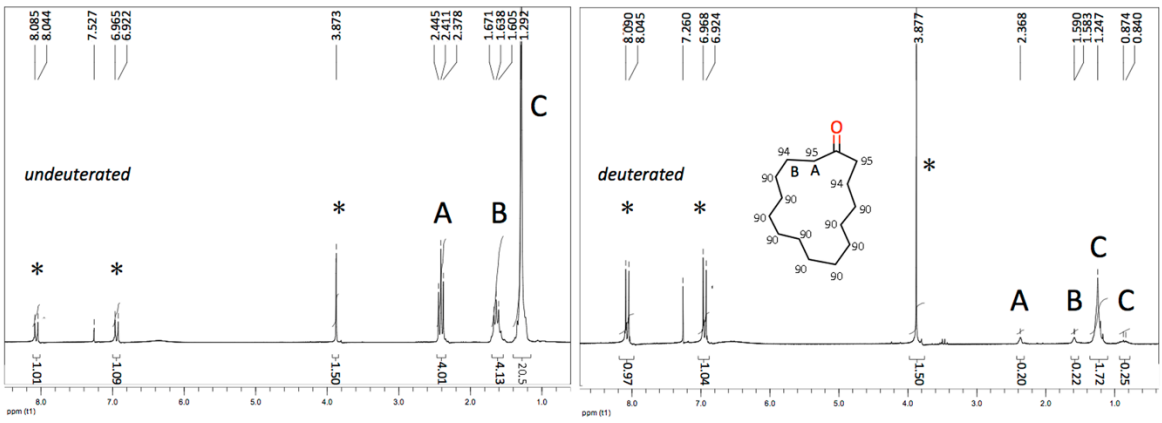



*Figure 57: NMR spectra of deuterated cyclopentadecanone in $CDCl_3$: Left: H- cyclopentadecanone. The peaks indicated by asterisks are those of the internal standard, p-anisic acid. Protons can clearly be resolved into 4xA, 4xB and the remainder [20 protons]. Right: Deuterated cyclopentadecanone. The 1H signal is greatly reduced. From the ratio of the integrated 1H*

*signal before and after deuteration we can calculate the yield to be 95% deuteration in the A position and 94% in the B position. The remaining protons are deuterated to 90%. No impurities are seen in the spectra. (From Gane et al (Gane et al. 2013))*

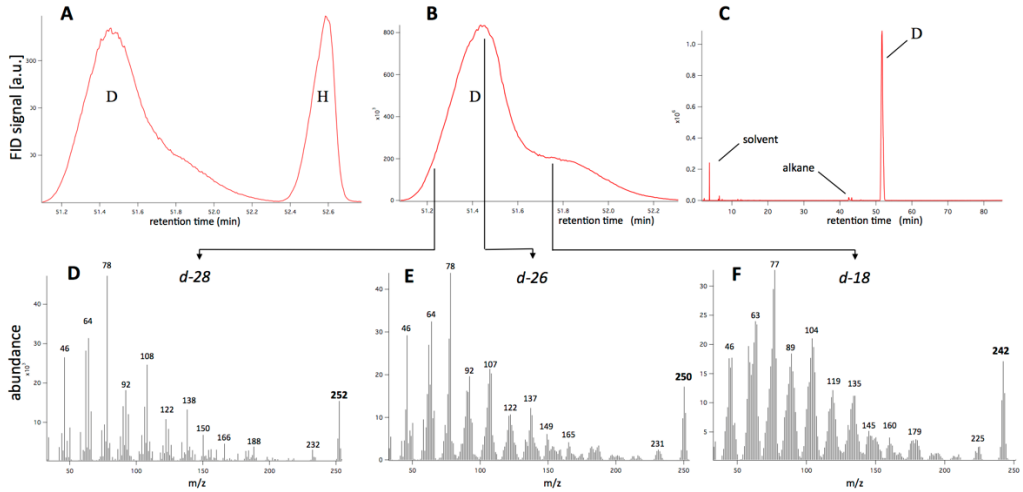



*Figure 58: GC and MS spectra of deuterated cyclopentadecanone. A: GC trace of a deliberate, approximately equimolar mixture of deuterated [D] and undeuterated [H] cyclopentadecanone. Good chromatographic separation is obtained, with a difference in retention time of approximately 1.2 minutes. B: GC trace of a different sample of deuterated cyclopentadecanone, coupled to mass spectrometry. C: Full GC trace of a sample of deuterated cyclopentadecanone after silica gel purification. The solvent peaks at retention times <15 min amount to 5% of total, and the alkane peaks [identified by MS] at 42–43 minutes amount to less than 1%. Peak D is then repurified by preparative GC and used in the smelling tests. D, E and F: Mass spectra obtained by sampling B at 51.21, 51.42 and 51.76 minutes. The units of the abscissa are daltons/unit charge. Consistent with panel A where the deuterated cyclopentadecanone exits the column before the undeuterated Exaltone, the perdeuterated species [d-28] exits the column first followed by less deuterated d-26 and d-18 fractions. (Images from Vioryl SA taken from Gane et al (Gane et al. 2013))*

After silica gel purification, aliquots of the deuterated musks were diluted in ethanol and trained odour assessors assessed their odour character on smelling strips. The parent compounds have classic powerful musk odour characters, with secondary descriptors as follows: animalic [cyclopentadecanone], sweet [Exaltolide®], oily [Astrotone®] and sweet [Tonalid®]. Several trained odour evaluators - Turin, and the fragrance chemists involved with the making of the musks at Vioryl SA (Nikitas Ragoussis, Dimitris Georganakis and Christina Koutsoudaki) independently assessed the novel deuterated molecules. In all the

deuterated musks the musk character was much reduced and a new character appeared, variously described by trained evaluators as "burnt", "roasted", "toasted", or "nutty". Subsequently in the study, naive subjects most commonly described the common character as "burnt".

Franco et al (Franco, Turin, Mershin & Skoulakis 2011b) showed that Drosophila were able to recognise a common "deuterated" character and to extrapolate this to other deuterated compounds. This "burnt" or nutty character may be the human version of this percept.

To control for any possible contaminants, I requested they make a series of "sham-deuterated" musks using exactly the same methods as above but using H2O instead of D2O. This ensured that any contaminants of the synthesis were likely to be found in both sample sets, reducing the likelihood of this being a method of distinguishing the two. These were used as the controls and smelled the same as the starting material, unsurprisingly.

**Purification**

Once synthesised the samples were transported to the trial site in London as relatively low concentration preparations. I used preparative Gas Chromatography as described above in the previous experiments to maximise purity and reduce the likelihood of identifying A/B by other cues or contaminants.

There are challenges to the GC purification of high molecular weight odorants. They have relatively high boiling points and therefore the entire length of the column needs to be kept above 180° C right until the sample collection. Early attempts at collection of the

sample with Eppendorfs were compromised by condensation of the musk at the exit of the sniffing port. I modified the GC to route the sample back out through the secondary injector port which allowed active heating until the point of sample capture.

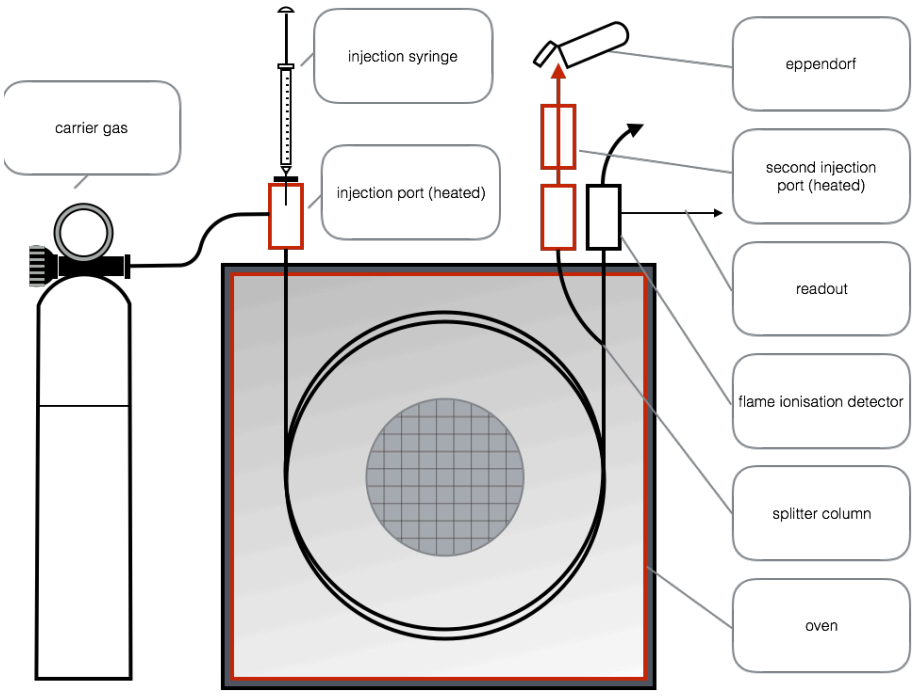



*Figure 59: Smelling GC modified for musks*

**Method (Temperature and Time)**

The column was maximally loaded with 10µl aliquots of increasing concentrations of solutions until the classic trace of column overload was seen. The concentration before this was then the standard concentration for both h- and d- forms of the odorant. Both the sham-deuterated and deuterated Exaltone were prepared for purification in the modified GC (described above) by dissolving to a 1 mg/µL concentration in 100% ethanol. The gas chromatograph settings were: oven at a constant temperature 190° C with the second

injection port at a constant 180° C, the packed column remained the same as previously. Since there were relatively low yields for each run, 4 runs were captured per tube.

To control for breakdown products and contaminants, a sample was captured into a clean Eppendorf and re-dissolved in 1 µL 100% ethanol, this was then reinjected approximately every 10 to 12 samples. The traces were identical apart from the volumes.

**Trace**

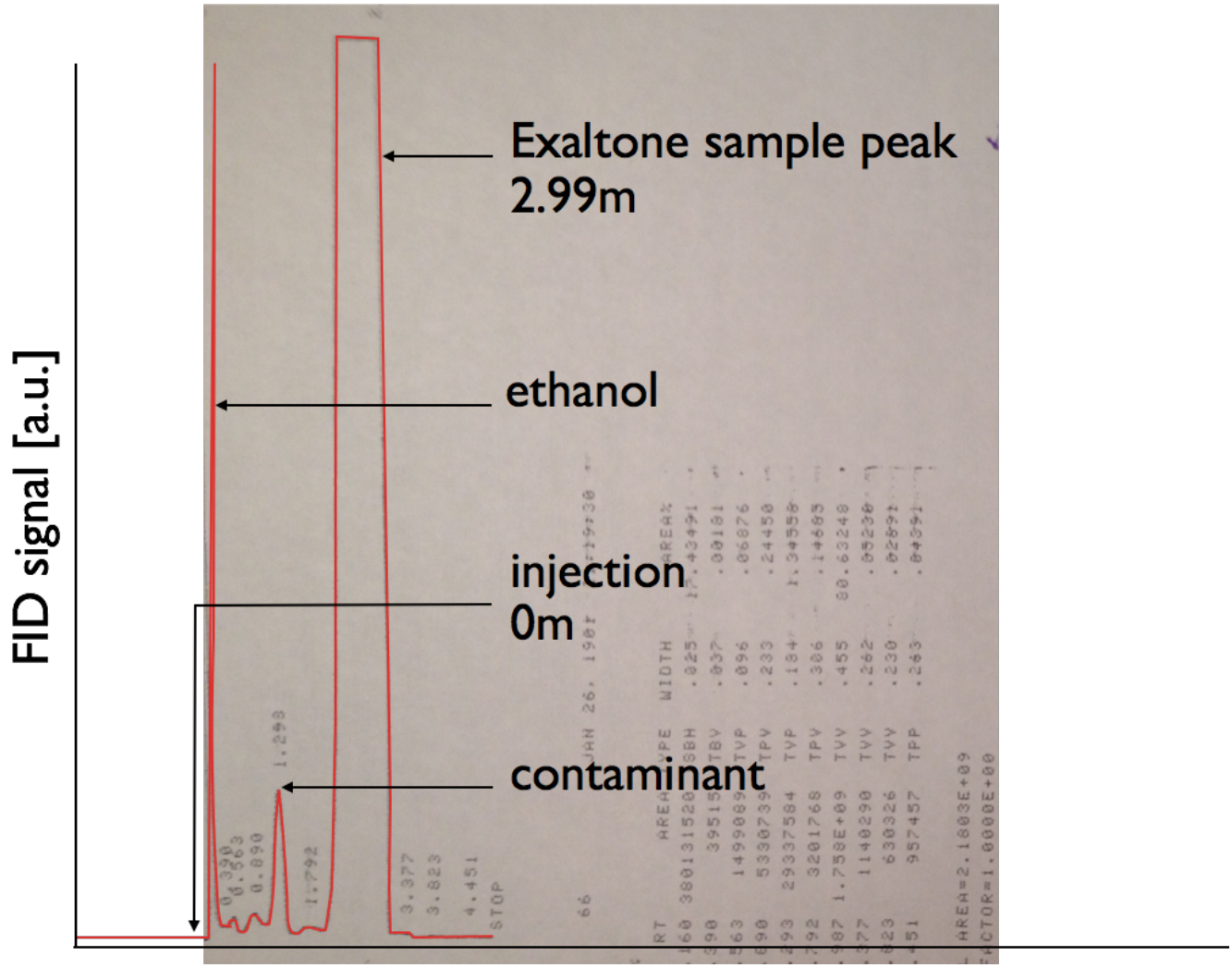



*Figure 60: Exaltone sample with printout. The fast transit of the highly volatile ethanol solvent is demonstrated with the large Exaltone sample peak at around three minutes from injection. There is artefactual clipping of the peak due to the scale of the detector line.*

**D-Exaltone**

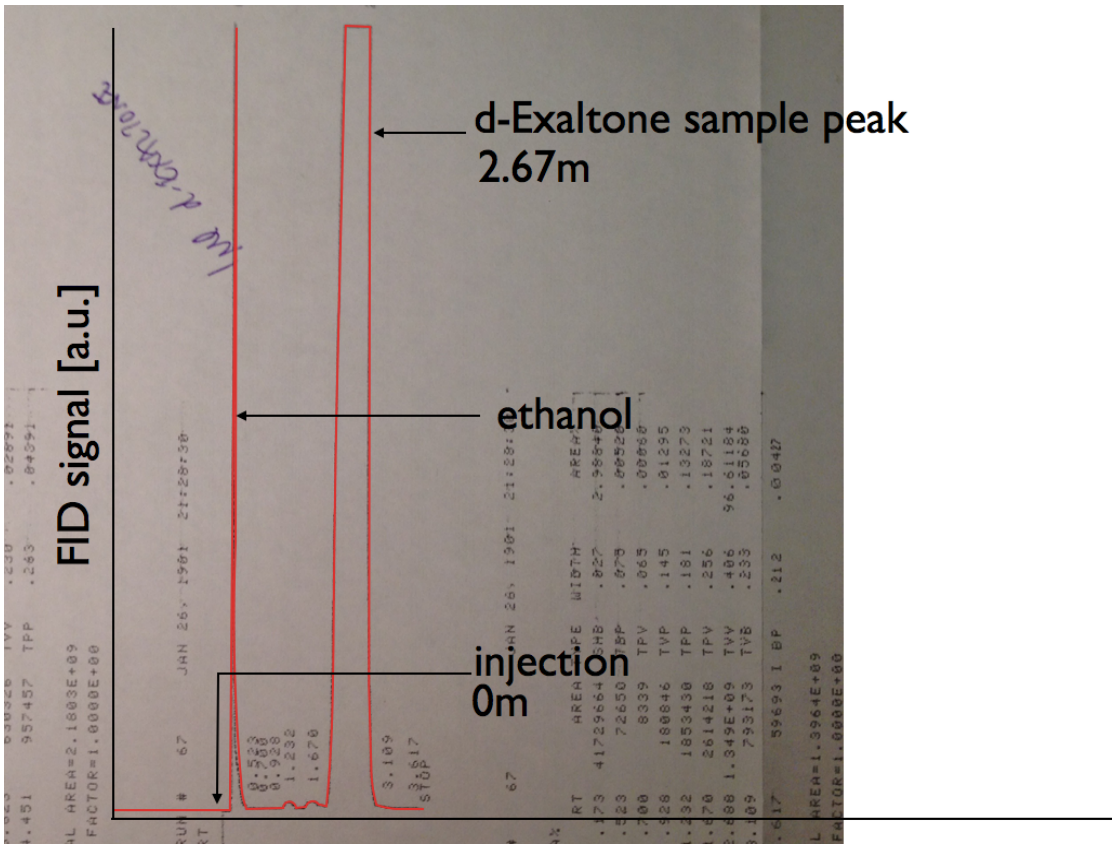



*Figure 61: d-Exaltone GC trace from printout, good separation and demonstration of lack of contaminant as this has already been purified in the Deuteration process described above this. The previously noted faster transit time of the deuterated Exaltone is recapitulated.*

**Preparation of Sample Tubes for Musks**

Standard capped Eppendorf LDPE tubes were coated internally with a thin layer of petroleum jelly by deposition from a mixture with low boiling point petroleum ether. This layer acted as an adsorbent to control the release of the odorant into the headspace.

To assess the retention of odour for the musks, several different substances were placed in the Eppendorf tubes and aliquots of non-deuterated cyclopentadecanone were decanted from the GC smelling port as in the standard method. Two tubes each were filled with cotton wool, glass wool, nothing and petroleum jelly.

The "wools" were approximately 1ml in volume and were chosen to significantly increase the adsorptive surface area.

The petroleum jelly layer was formed by the addition of a solution of commercial Vaseline® in an equal volume of low boiling point petroleum ether. 1 ml was added to the tubes and shaken, the excess was poured off and the ether allowed to clear at room temperature for 30 mins.

Two experimenters: one a trained odour assessor and one naive, both blinded, then assessed each sample for strength of odour, alteration of odour character and preservation of odour over multiple sniffs. Although there was no alteration in the odour character with any of the methods, the bare tube surface gave almost no odour after the first sniff. Both the cotton wool and the glass wool preserved the odours slightly longer, but they were not very strong. The petroleum jelly showed excellent retention of the sample odour across multiple trials with no alteration in odour character and no perceptible contamination.

It may be that a combination of the glass wool and the petroleum jelly deposition layer would be even better as the layer surface area would be vastly increased, but the simpler method worked well and was used as the standard preparation of all musk samples.

To double blind the experimenter and volunteer, each Eppendorf was labelled with a UV-visible ink. Each sample was assigned a unique identifier number for that trial, marked on the cap of the Eppendorf tube. The number was not visible in normal light, but under a portable UV light source (Helix) the sample number could be identified. The samples were thus indistinguishable to both experimenter and volunteer until after the volunteer had smelt and handed them back to the experimenter. The number could then be recorded, and the nature of the sample known.

**Randomisation**

In contrast to the acetophenone experiments, where a total of 20 samples were shaken in a box and selected a presented one at a time, in the musk trials, because many more GC passes were required to produce enough to give a strong smell signal from the Eppendorf, only four samples of each were made. However. I made three sets of these over the course of the experiment, to compensate for the rapid exhaustion of the samples. Additionally, the low volatility of the musk required that the Eppendorf tubes were warmed in a solid heating block to 50C during the experiment, which meant that I could not randomise and present the samples for each trial, as with the acetophenone randomisation method.

For this experiment, I arranged the samples in two rows of four within the heating block, one row of the deuterated and one of the non-deuterated. I then randomised the order of the tubes within the rows by means of two flips of a coin (first flip: assign the tube to first or second half of the row if both positions are open use a second flip: first or second spot within those; repeat until all are filled). The rows were then mixed randomly by a further coin flip per d/H pair (heads: swap positions, tails leave in situ). This randomised both the order in which the subjects were smelling the odorant but also which odorants were compared to which.

I watched the experiment through a window, not visible to the subject. The subject, eyes closed and wearing nitrile gloves to avoid contamination, smelt first one and then the other sample. Because the subject was left on their own, there was no way to physically blind them as in the first trials - they had to manipulate the samples themselves. Multiple sniffs at each sample were allowed. The subject was asked to identify the deuterated sample and to place it to one side. However, the UV labelling technique and identical Eppendorf tubes reduced the chances of identifying the samples by visual cues to

essentially zero. After four comparisons (between eight samples), I re-entered and placed the samples under the UV light source and identified them. The subject was not informed of the outcome. To avoid habituation the subject then rested their nose for 15 minutes before attempting the next trial. The samples were re-randomised and the test started again.

**Statistical analysis**

Once again, the initial experiment was to determine whether any single subject could robustly accomplish a task which should be impossible if the "shape" hypothesis is true: detecting the difference between molecules whose shape is the same although the vibrational modes are different.

A repeated 2 alternative forced choice measure of identification of the musks allowed easy randomisation and presentation to subjects, although the 50% threshold for random guessing would require a large effect size for significance at this sample size. Since the experiment is a binomial experiment, exact binomial tests are appropriate to assess the likelihood of a significant result.

In the event, most of the subjects were successful in the task and I performed a group analysis, assuming a normal distribution of the scores and a one-tailed t-test (since the question is whether the results are significantly higher than chance) can be used to test for significance.

In selecting a confidence interval, the hypothesis is a very contentious one and although not adopting a strictly Bayesian approach, the priors were very much in favour of the null hypothesis. Because of this I would reject the null only above 99%, requiring that the test be constructed so that repeated tests would only show a different result by random chance one in one hundred times.

**Power Calculations**

The initial methodology for the psychophysics experiments was described by Turin (pers. comm) as "demonstrating the possibility" of vibration detection in humans. It did not matter how many cannot smell the difference between deuterated and non-deutrated compounds, as long as the ability was robustly demonstrated in at least one. "It only takes one to fly to demonstrate that levitation is possible" as he said. I therefore did not perform a power calculation prior to the experiments.

## Genetics

The vibrational hypothesis is a description of the physics of the OR-olfactant interaction at the atomic level and the mechanism of GPCR activation.

The preliminary step in assessing the credibility of the theory, and a valuable way to identify specific case studies to investigate more rigorously. The previous work demonstrated the alteration of the odour character of Exaltone in humans; the next step is identify the responsible receptor to allow further work to confirm that this is a receptor-level phenomenon.

Identifying the receptor that is primarily responsible for the detection of the musk Exaltone would allow the closer interrogation of its function, and therefore more evidence for, or against, the vibrational hypothesis.

The process of linking an odorant to the receptor which detects it is known as deorphanisation and of this writing five olfactory receptors have been identified and fully validated with their ligands in humans.

The technical strategies used for this deorphanisation of the olfactory receptors can thought of in four broad categories: *in vivo*, *ex vivo*, *in vitro* and *in silico*. Most of these techniques are really only applicable to experimental animals.

In humans, deorphanisation usually relies on the natural experiment of *specific anosmias*. As opposed to a total smell-blindness: global anosmia, specific anosmias can be equated to colour-blindness, where the sufferer lacks the ability to detect the odour at all. Other individual variations in the threshold for an odorant are also useful in the investigation of the olfactory sense such as significantly reduced threshold (specific hyposmia) or raised threshold (hyperosmia) for detection. I propose, and use below, the term "specific threshold variant" (STV) as a catch-all for these. This does not include the more difficult-to-measure variation in valence (pleasantness), perception of odour intensity, or character although these are undoubtedly affected by individual OR variants, as discussed below.

The terms hyp-, hyper- and an-osmia are relative to the performance in perception against a norm. That norm is probably the background prevalence of the functional receptor: if the receptor is common in the population, those not expressing it will be said to have a hyp- or an-osmia. Conversely in a population, most of the members of which have a non-functional receptor, those with a functional receptor will have a hyperosmia. This can obviously vary between populations. The term "STV" captures both of these states without requiring any knowledge of the typical genotype. Historically, however, the literature usually refers to one state or the other, and I have used those terms as appropriate to the papers under discussion.

Specific anosmias have been used to examine the human olfactory system since Amoore pursued them as a "key to the odourcode" in the sixties and seventies (Amoore 1967). He developed a "stereochemical theory" (Amoore 1963) attempting to describe all odours by their relations to seven "prime" odours (seven dimensions must have seemed adequate or even generous compared to the three for vision, at least until the description of the real olfactory receptor genome at over 400 functional receptors).

In the pre-genomic era, specific anosmias were described to hydrogen cyanide (Kirk & Stenhouse 1953), n-butyl mercaptan (Patterson & Lauder 1948), isovaleric acid (Griff & Reed 1995b), β-ionone (Plotto et al. 2006), 1-pyrroline (Amoore et al. 1975) and others. They were also used to investigate the heritability of olfactory ability in humans (Wysocki & Beauchamp 1984) (Hubert et al. 1980) (Whissell-Buechy 1973) (Wysocki & Beauchamp 1984).

Although this heritability indicated a genetic cause for the STV, it was only with the demonstration of a specific anosmia to isovaleric acid in a laboratory mouse (strain C57BL/6J) (Wysocki et al. 1977) that brought the ability to map the genetic locus responsible: *Iva1*, to chromosome 4 (Griff & Reed 1995b). Using an early mouse reference genome this locus was shown to be a cluster of OR genes (Xinmin Zhang & Firestein 2002) which lent credence to the supposition that this recessive characteristic was receptor dysfunction.

STVs are the natural experiment in human receptor variation required to demonstrate the role of a receptor in human olfaction. In the genomic era Keller et al (Keller et al. 2007) were the first to demonstrate OR7D4 was responsible for a significant part of the human ability to smell androstenone with Menashe et al (Menashe et al. 2007) publishing a description of a hyperosmia to isovaleric acid shortly after associated with an association with a pseudogenising SNP in the OR11H7.

These papers laid the pattern for deorphanizing a human receptor in the era of single gene sequencing:

1. identify a phenotype: the specific threshold variant (STV)
2. identify a likely OR gene

3. sequence the target gene

4. show correlation between the sequestration of the genotype and phenotype

5. confirm the activity, or lack thereof, *in vitro*

I used this pattern to attempt to identify the musk receptor involved in the psychophysical experiments *supra,* as shown in Figure 62. At the beginning of this project, no musks receptors had been identified at all (Shirasu et al identified the first in 2014 (Shirasu et al. 2014)), but during the previous work I identified several musk anosmic volunteers. The existence of a musk STV was very likely to be the result of a single receptor loss in the affected subjects as discussed above. Analysing their genomes would allow the identification of that receptor if it were a causative pseudogenising mutation. By sequencing the families, I could identify more anosmics, examine inheritance patterns and reduce the number of variant genes to examine since all of the genes in one generation are also present in the previous one.

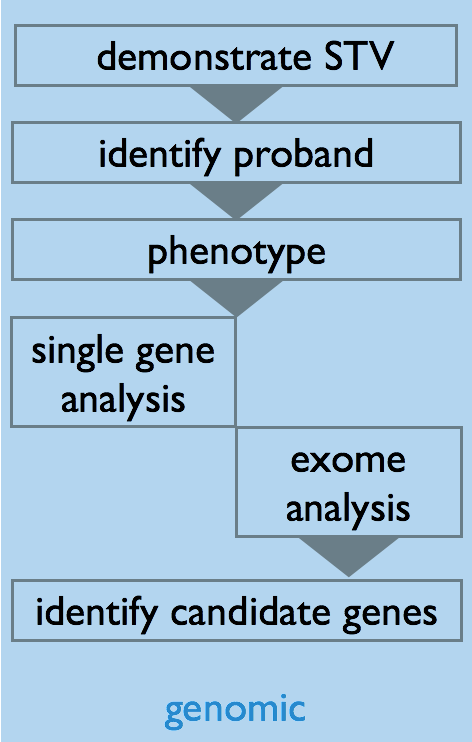



*Figure 62: Outline of genomic part of PhD project*

**Design**

Analysis of the genotype, either single gene or olfactory exome and comparison with psychophysical phenotyping of family members, some which were suspected to have a specific anosmia to musks. Family members are segregated by phenotype into musk osmics and anosmics. Variants that occurred in one group but not the other would be candidates for further investigation by expression and ligand profiling.

**Setting**

Phenotyping: psychophysical assessment at volunteer's home or in research institute laboratory space.

Genotyping: laboratory study at the Wellcome Trust Sanger Institute.

**In/exclusion Criteria**

All volunteers gave informed consent after reading the information sheet provided (see Ethical considerations, below). All subjects spoke English, but an interpreter was

available to explain any complicated points for those for whom English was not a first language. All first-degree family members of the index who was suspected to have the musk anosmia who gave consent for phenotyping and genetic sequencing were included. Olfactory ability was assessed but was not an exclusion criterion.

**Outcome Measures**

Phenotypic outcome measures were the demonstrated specific anosmia for the tested musks: I used three methods for pragmatic reasons, as described below:

1. Self-reported yes/no

2. Olfactory threshold to Galaxolide and Exaltone

3. Triangle testing for 50% Galaxolide

Genotypic outcome measures were the variant genes identified by:

1. Sanger sequencing a single gene (OR5AN1)

2. receptor-exome-wide sequencing.

**Phenotyping Familial Musk Anosmias**

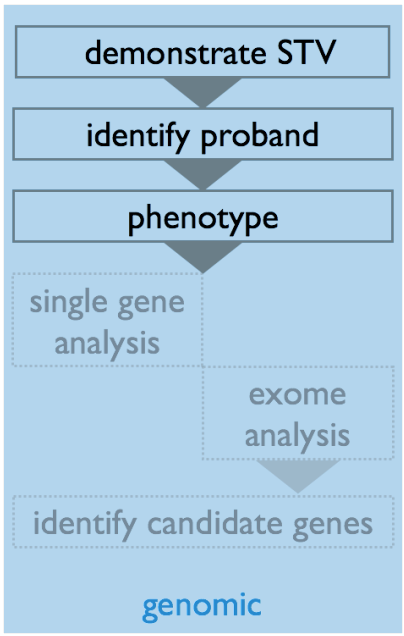



*Figure 63: Outline of genomic part of PhD project: phenotypic analysis*

**Participants**

During the first trials of deuterated musks, we identified a volunteer who could not detect any of the musks we were screening for use as a probe molecule. He reported a family trait of musk anosmia. Most of his immediate family agreed to further testing and genomic sequencing to attempt to elucidate a genetic basis for the anosmia. Another branch of the family was contacted but declined to participate in the trial.

Another co-worker also demonstrated a similar musk anosmia when asked. Although she did not know whether there was a family trait, her family agreed to be tested. A further volunteer was identified during the previous experiment but on further testing was shown to have a generalised poor olfactory ability but could smell the musk on repeat testing. One more co-worker at Vioryl SA self-identified as a musk anosmic and her family also agreed to be part of the study.

All families were ethnically Greek, and most family members were living in Athens during the study.

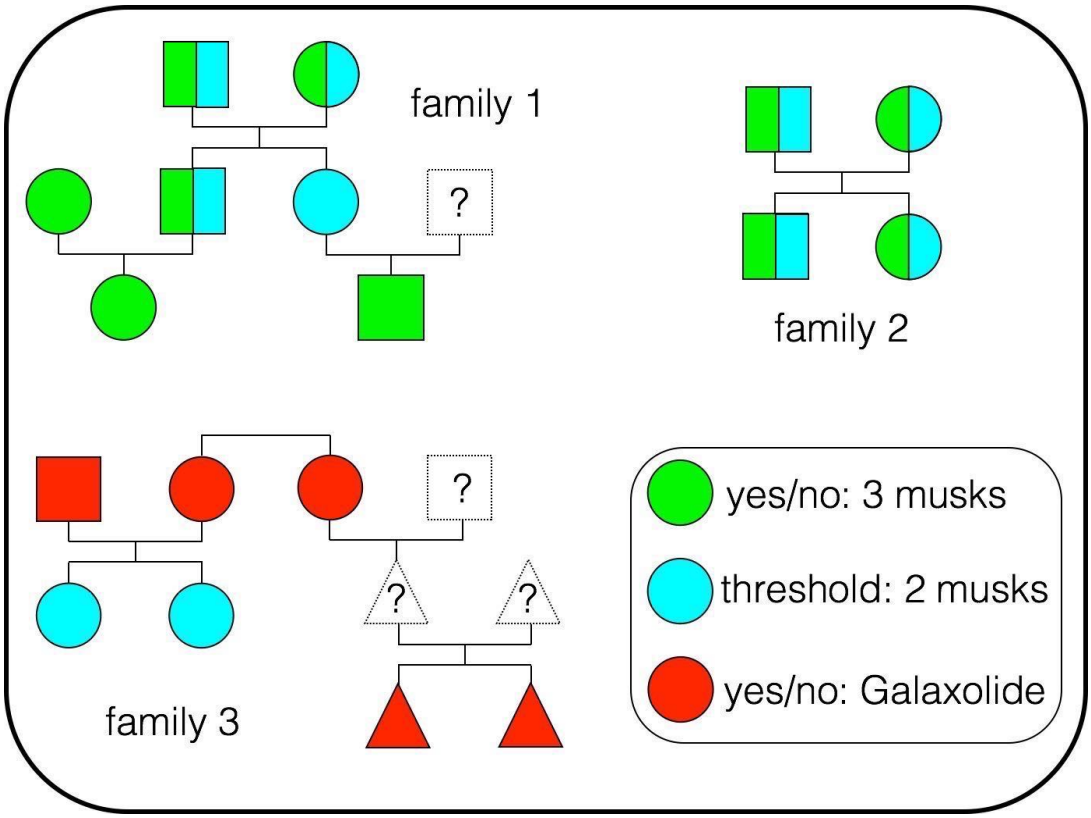
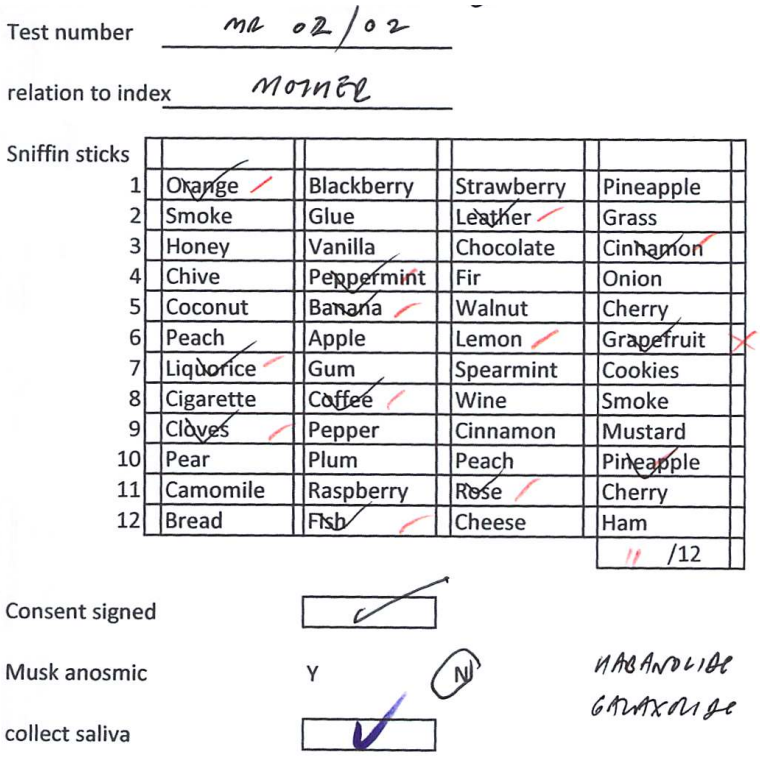



*Figure 64: Phenotyping methods employed for three families*

**Screening**

Subjects were asked not to eat or drink anything for at least an hour before the tests. All completed a questionnaire and 12-item supra-threshold Sniffin Sticks olfaction assessment (Hummel et al. n.d.).

The results were anonymised by physically cutting the top section of the results page, with the identifying information, away and stapling this to the consent form. This was the key if any de-anonymisation were required.



Test number MR 02/02

relation to index MOTHER

Sniffin sticks

| | | | | |
|---|---|---|---|---|
| 1 | Orange | Blackberry | Strawberry | Pineapple |
| 2 | Smoke | Glue | Leather | Grass |
| 3 | Honey | Vanilla | Chocolate | Cinnamon |
| 4 | Chive | Peppermint | Fir | Onion |
| 5 | Coconut | Banana | Walnut | Cherry |
| 6 | Peach | Apple | Lemon | Grapefruit |
| 7 | Liquorice | Gum | Spearmint | Cookies |
| 8 | Cigarette | Coffee | Wine | Smoke |
| 9 | Cloves | Pepper | Cinnamon | Mustard |
| 10 | Pear | Plum | Peach | Pineapple |
| 11 | Camomile | Raspberry | Rose | Cherry |
| 12 | Bread | Fish | Cheese | Ham |
| | | | | 11 /12 |

Consent signed

Musk anosmic Y N HABANOLIDE GALAXOLIDE

collect saliva

*Figure 65: Sample of anonymised phenotype results*

**Self-reported yes/no: Sample Preparation and Presentation**

The initial phenotyping of musk anosmia was a simple binary yes/no question, using perfumery-grade purest available samples donated by Vioryl SA. Each sample was in a cleaned and de-odourised standard glass perfumery bottle. Three musks were used: musk ketone, habanolide, and Galaxolide. Each was diluted in alcohol to 50% w/v.

To present the prepared samples, the bottles were opened and presented to the subjects and they were asked, "can you smell anything". If the respondents claimed to be able to detect an odour when the musk was presented, two trials of a triangle test were used to confirm this. This crude method would detect a pure musk anosmia, i.e. a complete inability to smell the substance, but would not demonstrate an altered threshold or reduced ability to detect an odour. Since the odorants were also presented in an odiferous medium, there may have been some false positive detections where the subject smelt the alcohol and

not the musk. Never having smelt the musk, they might not be able to recognise the odour of alcohol alone.

Nevertheless, there were several clear anosmics within the families, but a more robust method for identifying the true musk anosmic members was required.

**Threshold Testing for Galaxolide and Exaltone**

Consequently the thresholds were retested in the original subjects using a standard protocol as described by Gilbert et al (Gilbert & Kemp 1996). I designed the trial methodology, but due to travel constraints, the sample preparation and testing were performed by Ms Klio Maniati, a graduate student at the Alexander Fleming institute in Athens in the department of Prof E Skoulakis, a co-author on the previously published psychophysics results paper. Quality assurance is through her experience in the preparation and delivery of olfactory psychophysical testing as it forms part of her doctoral work, although these experiments are not part of her project.

Sample Preparation and Presentation

Samples were prepared from perfumery-grade musks generously donated by Vioryl S.A., Galaxolide at 50% and Exaltone at 97%. Both musks are solid at room temperature. Both odorants were diluted in diethyl phthalate in a binary dilution series, producing series of diluted samples with from 50% to a lowest concentration of 0.05%, a standard step dilution in olfactory psychophysics (Engen 1964). Samples were prepared in standard perfumery bottles, cleaned and dried as before. The negative control was diethyl phthylate only. For each sample, a perfumery blotter "smelling strip" was dipped and dried for 20 minutes to allow excess solvent to evaporate.

Prior to the test, subjects smelled both the control odour and musk odour at 50% to demonstrate the difference between the two. Several of the subjects could not smell the

Galaxolide even at the maximum concentration. In the test, the first presentation was of the middle concentration step: 0.5%

Threshold Determination

A 2-alternative forced-choice (2 AFC) test was used to elicit the subject's thresholds for the musks with ascending and descending limits with up to seven reversals (Gilbert & Kemp 1996). In some older subjects seven reversals were not tolerated and reversals were stopped at four. On presentation of the first sample pair in random order the subject then indicated by pointing which of the two strips were the musk. If the subject could not decide they were asked to guess. If the answer was correct, the next lower dilution step was presented. If the answer was incorrect, the sample pair was re-presented. After two incorrect answers, the next higher concentration step was presented and so on until two correct choices were made. The next lower dilution step was then presented, this counted as a reversal. The threshold was calculated as the lowest correct dilution step after the seven reversals

**Galaxolide Triangle Screening**

For supra-threshold triangle screening the highest concentration (50%) of Galaxolide in diethyl phthalate was used to dip standard perfumery smelling strips. The diluent diethyl phthalate was the control. A series of three failed triangle tests in a row was considered anosmia (cumulative chance if random ~3%). The subject's own report of being able to detect the odour was confirmatory evidence. There were no cases where the two were in conflict although the sample size was small.

**Genotyping: Single Gene Analysis Methods**

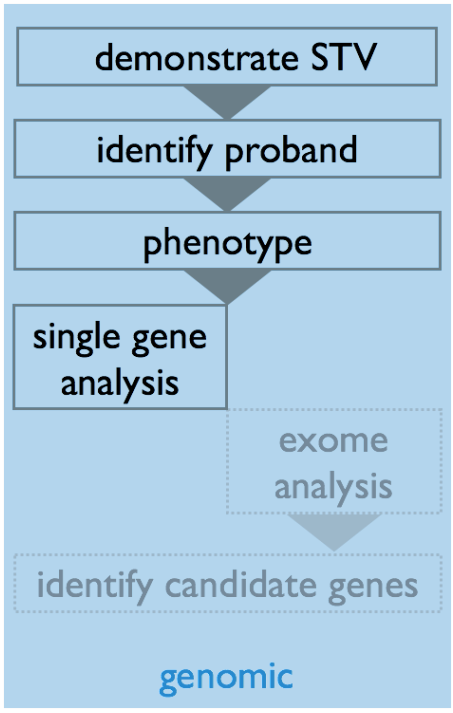



*Figure 66: Outline of genomic part of PhD project: single gene analysis*

Given the inheritance patterns it is possible that the anosmia is due to an autosomal dominant single gene, although pseudo-dominant and recessive patterns are also possible small size of the sample. A single pseudogenised musk receptor might explain the phenotype.

Shirasu et al (Shirasu et al. 2014) identified the mouse musk receptor MOR215-1 and demonstrated fairly clearly that this was a narrowly tuned musk receptor. They cloned the human homologue OR5AN1 and demonstrated activation in a HEK293 expression system by muscone but did not examine it further.

As shown in Figure 66 I investigated the possible single genetic cause of the familial musk anosmia in my cohort, even if they demonstrated an anosmia to Galaxolide and not to Exaltone.

**OR5AN1**

Found on chromosome 11 (region 59,364,412 -59,365,448 in GRCh38 coordinates), a region with which also contains the genes for 11 other olfactory receptors, OR5AN1 is a 1kB single exon gene sharing the DRY motif as described by Axel and Buck (Buck & Axel 1991) and recognised to code for a human olfactory receptor. It has a 68% amino acid homology with the mouse OR215-1 and has been shown *in vivo* to be activated by muscone (Shirasu et al. 2014). It did not have any detected odorant responses in a recent large scale high-throughput analysis (Mainland et al. 2015).

The gene has two paralogues: OR5A1 and OR5A2, with the most similar paralogue being OR5AN2P with a sequence similarity of 67.21% (HORDE database information (Anon n.d.)).

**DNA Samples**

For all volunteers who gave permission, I collected genomic DNA in Oragene•DNA™ (OG-500) saliva sample kits (DNA Genotek, Inc., Ottawa, Ontario, Canada) as per the kit instructions. These provide DNA comparable to blood samples for genomic DNA (Nunes et al. 2012),(Rylander-Rudqvist et al. 2006) and collection is well tolerated. The samples were collected in two tranches. One volunteer's sample (01/03) from the first tranche was recollected the second tranche due to the poor quality of the initial sample. The tranches are shown in Table 4 and Figure 67.

Only tranche 1, the sequences for families one and two, were available for analysis for this portion of the experiment.

| *family* | *volunteer* | *relation* | *DNA tranche* |
|---|---|---|---|
| *1* | 01.01 | index | 1 |
| | 01/02 | father | 1 |
| | 01/03 | mother | 2 |
| | 01/04 | nephew (sister's son) | 1 |
| | 01/05 | wife | 1 |
| | 01/06 | daughter | 1 |
| | 01/07 | sister | 2 |
| *2* | 02/01 | index | 1 |
| | 02/02 | mother | 1 |
| | 02/03 | father | 1 |
| | 02/04 | brother | 1 |
| *3* | 03/01 | index | 2 |
| | 03/02 | sister | 2 |
| | 03/03 | mother | 2 |
| | 03/04 | father | 2 |
| | 03/05 | maternal aunt | NA |
| | 03/06 | niece | NA |
| | 03/07 | niece | NA |

*Table 4: Three recruited families demonstrating relations to the index volunteer and tranche of DNA collected.*

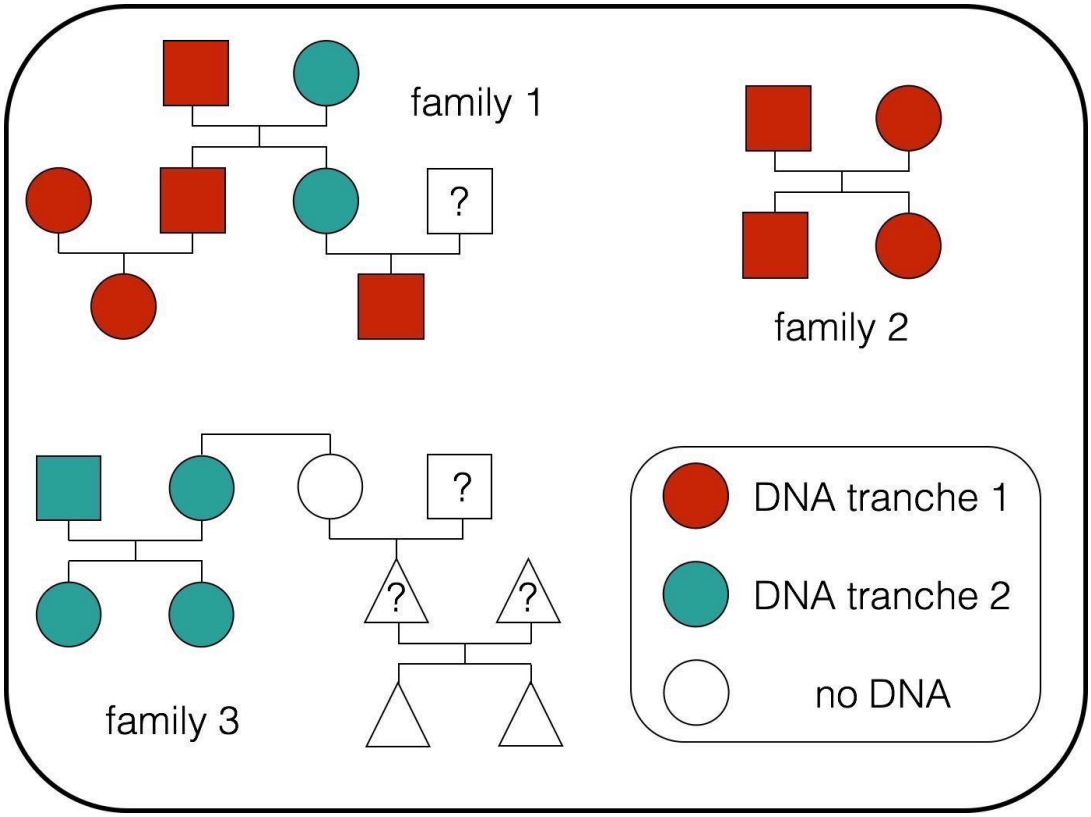



*Figure 67: Genogram with all three families, showing DNA tranches collected.*

Unfortunately, the Sanger Institute was only able to sequence the first tranche and this is what has been made available for bioinformatic analysis thus far.

The calculated concentrations for the first tranche of musk samples is given in Table 3. DNA concentrations were obtained by suspending the samples in 1X TE buffer and placing in a quartz cuvette. They were then calculated with a spectrophotometer comparison of absorbance at 260nm against that at 320nm in the standard method.

| volunteer | DNA concentration |
|---|---|
| **01.01** | 255 ng/µL |
| **01/02** | 392 ng/µL |
| **01/03** | 600 ng/µL* |
| **01/04** | 103 ng/µL |
| **01/05** | 231 ng/µL |
| **01/06** | 76 ng/µL |
| | |
| **02/01** | 488 ng/µL |
| **02/02** | 213 ng/µL |
| **02/03** | 51 ng/µL |
| **02/04** | 300 ng/µL |

*Table 50: Salivary sample yield of DNA from first tranche of volunteers.*

The highest concentration was volunteer 01/03, which may indicate a spurious result as this sample was shown to be insufficient on single gene and whole exome methods.

**Amplification**

To investigate whether the putative human musk receptor OR5AN1 was responsible for the musk anosmias in the families, I obtained the structure from the Ensembl genome browser (www.ensembl.org) website and using the primer3 site

(www.primer3.uu.ee), designed two pairs of primers. Sanger sequencing is usually able to cover about 500Bp, Therefore since the gene is over 1Kbp two sets of primers were constructed: one pair from the ends and one pair nested within the middle of the gene, from sense and antisense strands. These primers are shown in Table 6.

| *Oligo Name* | *Sequence* |
|---|---|
| *Or5an1_F1* | *TGTCCATTTCCAGTGCTTGT* |
| *Or5an1_R1* | *GGGAGATTCTGATGGGCCAT* |
| *Or5an1_F2* | *CACCTATTTCATCCTGCTGGG* |
| *Or5an1_R2* | *TGTGCATCAAATCAAGCCTTCT* |

*Table 6: Forward and reverse primers for OR5AN1*

For the PCR amplification, I diluted each primer to 10µg/ml concentration. 2.5 µl of the forward and reverse primers were added to 25µl of ThermoPrime *Taq* DNA Polymerase with 10X ReddyMix PCR Buffer (ThermoFisher Scientific) and 1µl of the pelleted and resuspended DNA. The solution was made up to a volume of 50µl with distilled, nuclease-free water.

The PCR thermocycler settings were as presented in Table 7.

| *Step* | *Temp °C* | *Time* | *Number of cycles* |
|---|---|---|---|
| *Initial Denaturation* | *95* | *2 min* | *1* |
| *Denaturation* | *90* | *25s* | *40* |
| *Annealing* | *55* | *35s* | |
| *Extension* | *72* | *65s* | |
| *Final Extension* | *72* | *5 min* | *1* |

*Table 7: PCR Thermocycler settings (GRED58)*

**DNA Agarose Gel Preparation**

To confirm the successful amplification of OR5AN1 I performed DNA agarose gel electrophoresis according to the standard method at the Sanger Institute to prepare the samples for sequencing.

I poured a 1.5% agarose gel: 1.5g of agarose in 100ml TAE buffer (40mM Tris(hydroxymethyl)aminomethane, 20mM acetic acid, and 1mM EDTA) into a gel tray with a well comb in place. Once solidified, I placed this into the electrophoresis unit and covered with the TAE solution.

In a 32-well plate I placed two rows of: a 1kb "hyperladder" calibration solution, each of the twelve samples (01.01 to 02/04), a negative control, another calibration solution and a positive control of human cDNA HL60.

I ran the gel at 120V for 1 hr. I then stained the gel by placing it in a container with 100mL of TAE and 5 µL of EtBr (Ethidium Bromide) on a rocker for 30 mins, followed by a "destain" with 100mL of DI H2O for a further 5 mins. The plates were visualised using a BIORAD UV transilluminator camera, shown in Figure 68.

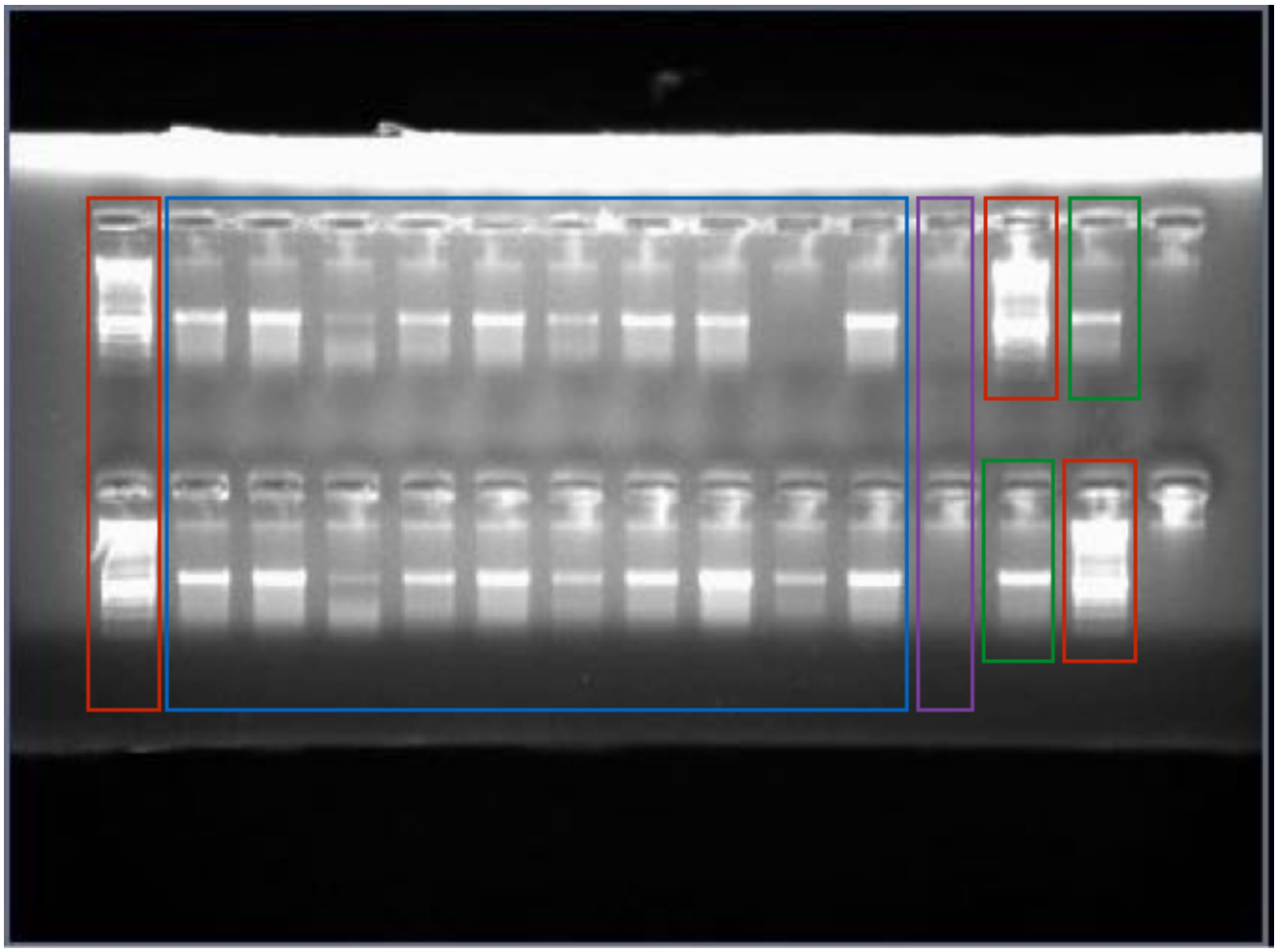


*Figure 68: BIORAD DNA agarose gel of PCR of OR5AN1 for families one and two. The top and bottom rows are the same samples. The coloured sections identify: red - hyperladder, blue – subject DNA, mauve – negative control (H2O), green – positive control (HL60). The poor quality of the DNA for subject 1.03 can be seen in the fourth column from the left.*

Having confirmed the presence of amplified DNA of the correct size (about 1kB), the samples were then excised and purified using the QIAquick Gel Extraction kit (QIAGEN) as per manufacturer's instructions and sent to the Sanger Institute pipeline for Sanger sequencing.

**Sanger Sequencing**

Described by Sanger and colleagues in 1977 (Sanger et al. 1977), Sanger sequencing uses a DNA polymerase enzyme with primers designed for the area under investigation in a mixture of normal DNA bases (alanine -A , guanine -G, thymine -T and cytosine -C) and chain-terminating bases. These bases are the 2,3-dideoxy versions of the deoxyribonucleotides: ddT, ddA etc. The DNA is incubated with the polymerase enzyme, which makes multiple copies of the region targeted by the primers. Each "run" of these copies will be stopped at a random point by the incorporation of a chain-terminating base, leaving a mixture of fragments of the gene of different lengths, ending at each base in the sequence.

In the original description of the technique, each base was run in a separate incubation, then fractionated by electrophoresis and the sequence then constructed by reading the gels together. In modern Sanger sequencing the terminating bases are labelled with a corresponding unique colour flourochrome. When separated in a gel, each flourochrome can be "read" by a laser reader and a linear graph of the colour versus the length of the fragment, a chromatogram, produced.

Sequencing was carried out at the Sanger Institute on a Illumina SBS (Bentley et al. 2008) using HiSeq v4 chemistry on a Hiseq 2500 according to manufacturer's instructions

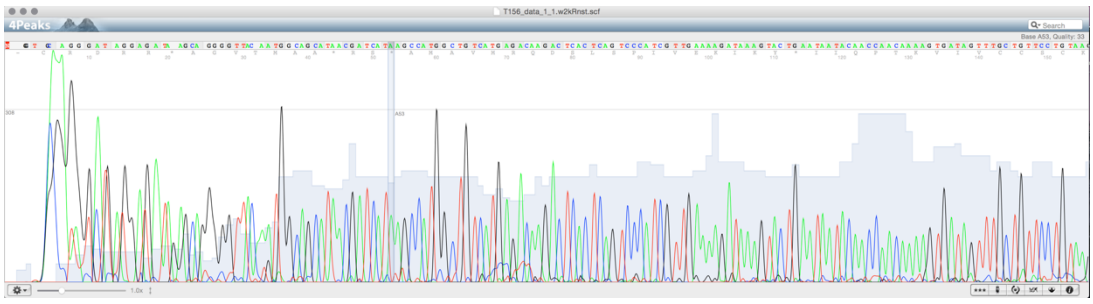



*Figure 69: Sample chromatogram*

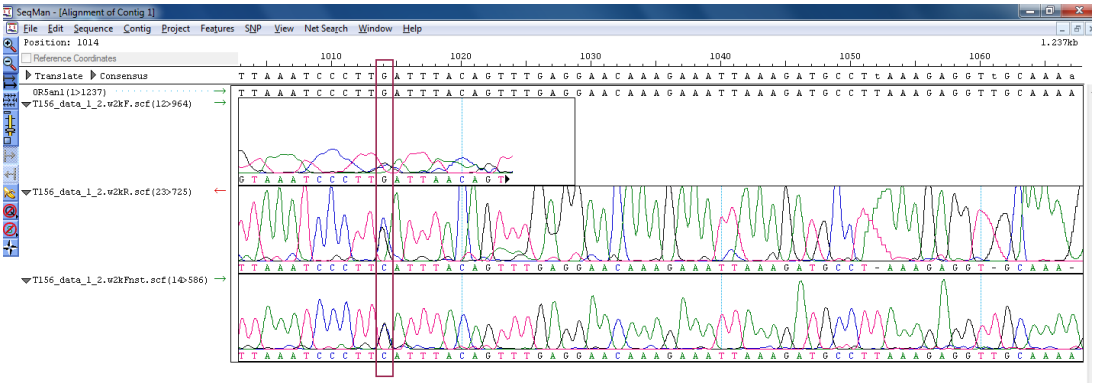



*Figure 70: Sanger sequence chromatogram and sequence alignment.*

Using SeqMan Pro software (DNAStar) the sequences were aligned to the known structure of the gene and manually reviewed for any variation. One sample (01/03) did not have enough coverage to determine the sequence of the gene and was repeated in tranche 2, which has not been sequenced yet.

## Genotyping: Exome Sequencing

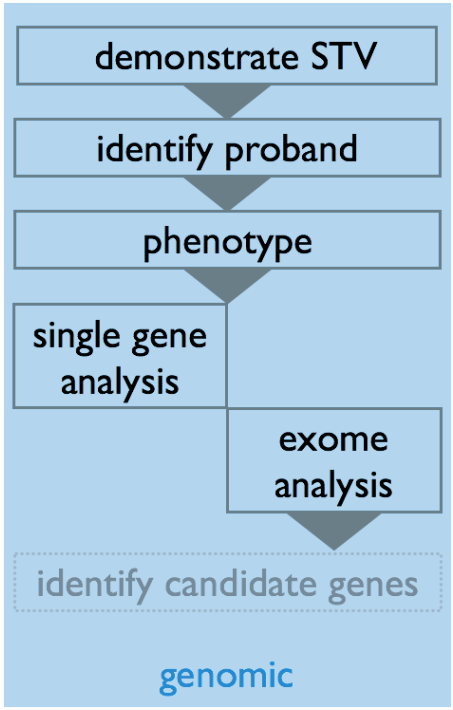



*Figure 71: Outline of genomic part of PhD project: exome analysis*

Single gene studies rely on examining one gene at a time, a more efficient approach would be to examine all likely causative genes and to look for relative enrichment in the affected versus un-affected populations. This statistical approach can, at the very least, narrow down the range of ORs to be further examined.

The approach taken for this project at the Wellcome Trust Sanger Institute and Monell Chemical Senses Center is similar. The subject's genomic DNA is sequenced by the Sanger sequencing pipeline and the raw sequence data narrowed down to the genes that are expressed as whole proteins - the exome. Initially we examined only the olfactory receptor exome, although a wider whole-exome analysis is also planned. The recognised variants were identified using the GATK toolkit (McKenna et al. 2010) (DePristo et al. 2011) then filtered based on the phenotype. OR genes that had a variant in only the anosmic or osmic sections of the family were identified. The sequencing was able to identify the variant allele and determine whether the variant was homo-or heterozygous in the subject.

Dr Casey Trimmer and Dr Darren Logan of the Monell Chemical Senses Institute and Wellcome Trust Sanger Institute performed the analysis and classification of pseudogenes with my input on phenotype; all the rest of the work is mine.

**Sequencing Methods**

The samples were sequenced on the Wellcome Trust Sanger Institute exome pipeline using the standard method for Illumina dye sequencing described below from information given by Dr S Austin-Guest of the Wellcome Trust Sanger Institute.

Tagmentation

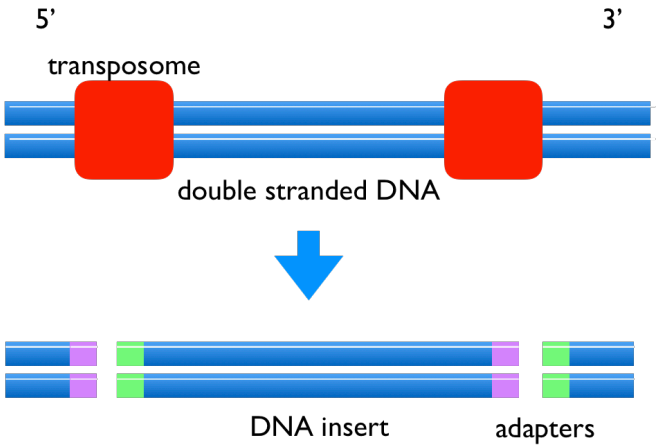



*Figure 72: Tagmentation step showing division of dsDNA by transposomes and terminal tagging of fragments with primer binding sites and adapters. Adapted from Illumina Sequencing Technology video by Illumina https://youtu.be/womKfikWlxM accessed 27/05/2018*

The first step of the sequencing is to fragment the DNA and ligate with adapters.

From the samples provided, 500ng of genomic DNA was fragmented (average size distribution ~ 150bp, LE220, Covaris Inc) and purified. Libraries were prepared with Agilent SureSelect XT custom kits (Agilent Technologies), and index tags from the Sanger 168 tag set applied.

Reduced Cycle Amplification

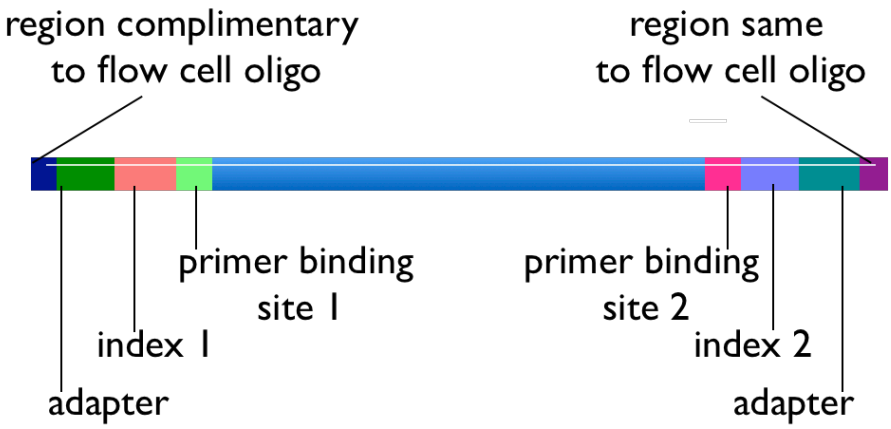



*Figure 73: Reduced cycle amplification: addition of indices, primer binding sites and terminal sequences to facilitate amplification. Adapted from Illumina Sequencing Technology video by Illumina as before*

These Index tagged samples were then amplified with 6 cycles of PCR using KAPA HiFi kit (KAPA Biosystems), quantified with a k assay using LabChip GX, (Perkin Elmer), then rearrayed and grouped into predefined pools, in an equimolar fashion based on quantitation value obtained from the assay.

Bridge Amplification

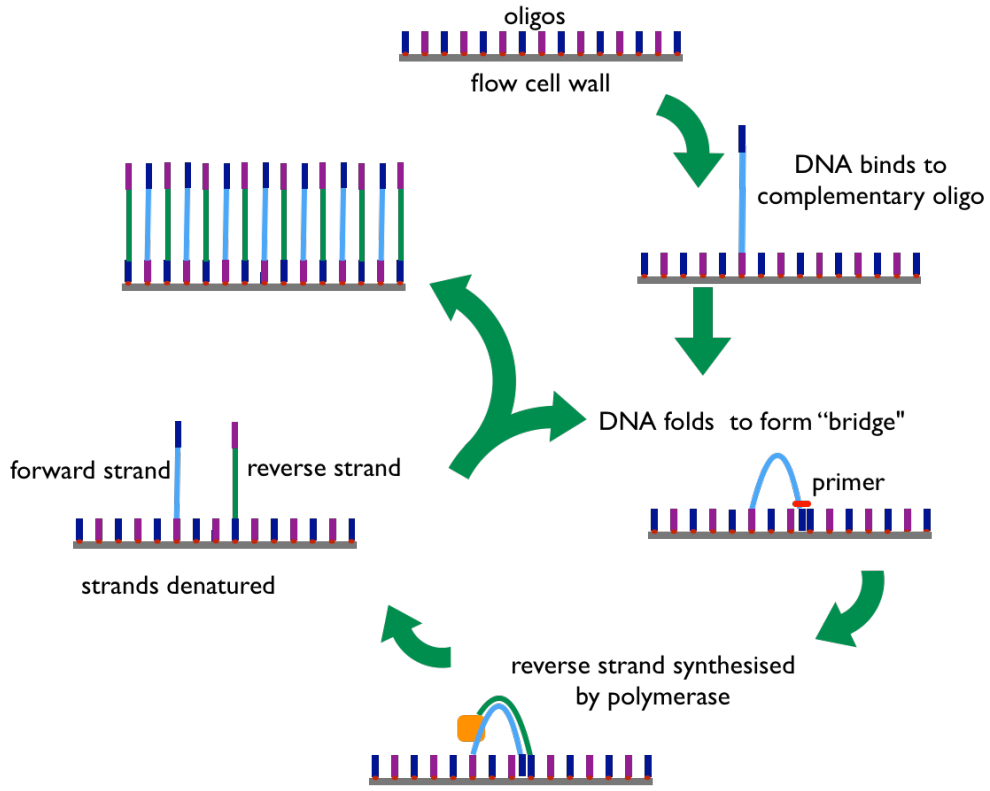



*Figure 74: Bridge amplification: the prepared strands attach to complementary oligos on the flow cell wall. The strands fold and bind to second oligo after which reverse strand is polymerased. These are then denatured, and the process repeats until there is a cluster of DNA forward and reverse strand clones. Adapted from Illumina Sequencing Technology video by Illumina as before.*

These pre-capture pools then underwent hybridization with a designed bait set: Human All Exon V (Agilent Sureselect Target enrichment Kit), with subsequent post capture wash (Agilent SureSelect Target enrichment kit).

Clonal Amplification

The pools then underwent a further 12 cycles of PCR for enrichment using Herculase II Fusion polymerase and were then purified (Ampure XP, Beckman coulter), quantified (1K assay, Bioanalyser, Agilent Technologies), normalised to a molarity of 6nM, and submitted to cluster formation for HiSeq V4 paired end sequencing (5 lanes, 75bp PE read length, Illumina inc).

**Bioinformatics Methods**

The bioinformatics analysis work was undertaken by Dr Casey Trimmer at the Monell Chemical Senses Center, a summary of her methods is given here:

The sequence variants were identified using a custom-made pipeline that followed the current best practices for variant detection developed by the Broad Institute (Van der Auwera et al. 2014; DePristo et al. 2011; McKenna et al. 2010).

The output from the sequencing reads were aligned to the human reference genome (version GRCh37) and PCR duplicates were removed with Picard Tools (Broad Institute 2012). The Genome Analysis Toolkit (GATK) was used to realign reads around insertions and deletions, recalibrate base quality scores, and genotype variant sites (Van der Auwera et al. 2014; DePristo et al. 2011; McKenna et al. 2010).

After variants were identified, the R statistical package (R Core Team 2016) was used to identify those variants occurring in a list of 668 genes with intervals, generated from previous unpublished work at Monell. Some non-olfactory receptor genes were added to this list because some previous work (also unpublished) showed their homologs to be

expressed in the mouse olfactory epithelium. This OR list was a standard list used by researchers at Monell and is given in Appendix B.

These variant olfactory receptors were then separated into those which were found in anosmics but not normosmics (loss-of-function) and variants found in normosmics but not anosmics (gain-of-function).

This process provided a list of candidate OR genes, their sequence variation and zygosity. The variants result in either missense (one or more amino acid substitutions) or non-sense (e.g. frame shifts or premature stop codons). Nonsense variants are obvious pseudogenes, especially if early in the nucleotide sequence, with a stop codon or frame shift (which will almost inevitably result in a stop codon downstream) the gene cannot form a normal receptor protein. Missense mutations are more difficult to label as obvious pseudogenes, one amino acid replacement may have no effect on a receptor's expression or functional selectivity but equally may be very important for one or both. Highly conserved amino acids are more likely to be necessary for normal function and variants at these sites may lead to an otherwise apparently normal gene being labelled as a pseudogene. A more formalised approach is embodied in the CORP algorithm (Classifier for Olfactory Receptor Pseudogenes) (Menashe et al. 2006), which uses a probabilistic approach to analysing the likelihood non-functional gene product by comparing the sequence deviation from a consensus of functional and highly conserved residues and assigning a score between 0 (functional) and 1 (pseudogene).

## Chapter summary

In summary the methods for achieving the objectives outlined in the literature review are in two strands: psychophysical and genetic. The psychophysical methods are

concerned with robustly showing the ability of some humans to detect a difference in the smell of deuterated molecules. The subsequent half of the methods are directed at identifying the receptor which is responsible for detecting the altered signal by correlating genetic variants with an inherited specific anosmia. These two will lay the groundwork for proving that the alteration demonstrated in the first part is a receptor-level process as predicted by the vibrational hypothesis.

# Chapter Three: Psychophysics Results

## Chapter Synopsis

I report in this chapter the results of the psychophysics experiments directed at achieving my first research objective: demonstrating an alteration of odour quality between two molecules, deuterated and undeuterated. I performed three experiments, a pilot experiment with unpurified toluene, an experiment with purified acetophenone using trained and untrained smellers and finally a test using untrained smellers to detect a musk odour alteration in highly purified samples.

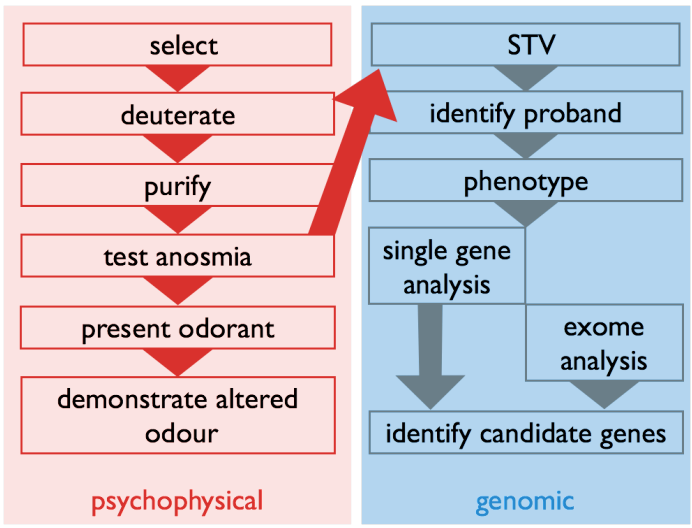



*Figure 75. Overview of the whole project, the psychophysical portion of which is presented in this chapter*

## Toluene Pilot experiment

As a pilot project, I used readily available unpurified toluene and deuterated toluene, with three volunteers.

**Results**

| VOLUNTEER | AGE | GENDER | NASAL SYMPTOMS |
|---|---|---|---|
| **MW** | 24 | M | None |
| **JB** | 25 | F | Allergic rhinitis |
| **SB** | 35 | M | None |

*Table 8: Volunteers for toluene assessment experiment. All were post-graduate students at UCL.*

The demographics of the sample group are presented in Table 8

| VOLUNTEER | SUCCESSES | TRIALS | % SUCCESS | LOWER BOUND OF 95% CI | UPPER BOUND OF 95% CI | P VALUE |
|---|---|---|---|---|---|---|
| **MW** | 9 | 15 | 60 | 32 | 84 | 0.60 |
| **JB** | 7 | 15 | 47 | 21 | 73 | 1 |
| **SB** | 9 | 15 | 60 | 32 | 84 | 0.60 |

*Table 9: Toluene results with binomial significance calculated with Pearson-Clopper exact method.*

Subjectively, after some practice 2 subjects (the same two scored highly) said they could begin to distinguish between the two samples. Although there was subjective improvement in their ability to detect a difference, the volunteers were no more likely to successfully identify the correct sample in the first half of the trial than in the second.

Objectively, two volunteers scored 9 correct out of 15 (60%) and the third with severe clinical rhinitis, scored 7 correct as shown in Table 9. Using a Pearson-Clopper exact method for binomial experiments, there is no strong evidence that any of subjects could detect a difference between deuterated and undeuterated toluene at a rate greater than chance.

All subjects reported that the odorant was unpleasant to smell and one (JB) reported a headache after the experiment, although no significant adverse events followed.

**Summary of results**

The small number of subjects in this experiment could not demonstrate that unpurified toluene has appreciably different odour character to its deuterated counterpart. The relative unpleasantness of the odorant meant that it was a poor choice for continuing the psychophysical experiments.

## Acetophenone

As discussed in the previous chapter, I chose acetophenone because of its availability and pleasant characteristics.

**Group analysis**

The results of the six recruited volunteers are summarised in Table 10 below.

| VOLUNTEER | AGE | GENDER | PROFESSION |
|---|---|---|---|
| DR | 42 | M | Perfumer |
| TG | 58 | M | Perfumer |
| TS | 47 | M | Perfumer |
| AD | 27 | F | Student |
| SG | 37 | M | Student |
| MS | 70 | M | Academic |

*Table 10: Summary of volunteer characteristics*

| VOLUNTEER | SUCCESSES | TOTAL TRIALS | SUCCESS % | LOWER BOUND OF 95% CI | UPPER BOUND OF 95% CI | P VALUE | TRAINING | PRO |
|---|---|---|---|---|---|---|---|---|
| **DR** | 39 | 80 | 49 | 37 | 60 | 0.91 | yes | yes |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **TG** | 44 | 80 | 55 | 43 | 66 | 0.43 | no | yes |
| **TS** | 172 | 350 | 49 | 44 | 55 | 0.79 | yes | yes |
| **AD** | 154 | 320 | 48 | 43 | 54 | 0.54 | no | no |
| **SG** | 217 | 400 | 54 | 49 | 59 | 0.10 | no | no |
| **MS** | 36 | 70 | 51 | 39 | 64 | 0.91 | yes | no |

*Table 11: Results with binomial significance calculated with Pearson-Clopper exact method.*

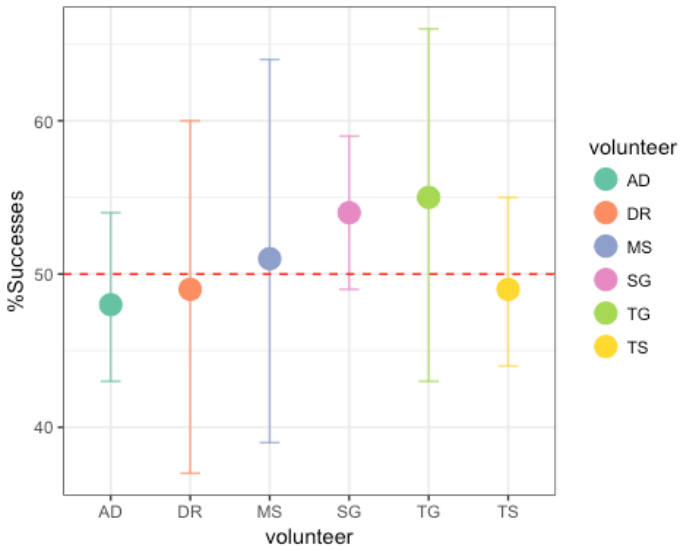



*Figure 76: No subject demonstrated discrimination above chance at p=0.05. Chance (50%) marked by dotted red line.*

To assess the significance of the trials I performed a one-sample, one-sided t-test, with the null hypothesis being that the ability to correctly identify the deuterated sample in a pair is not greater than chance (null probability = 50%).

The computed mean was 51, with a p value of 0.22 so we cannot reject the null and the sample mean is not greater than chance.

**Effect of Training Set on Success Rate**

Half of the volunteers (MS, DR and TS) were given a training set – two pairs of the samples, each identified and handed to the volunteer to smell as much as they liked, before undertaking the blinded experiment. The results are demonstrated in Figure 77.

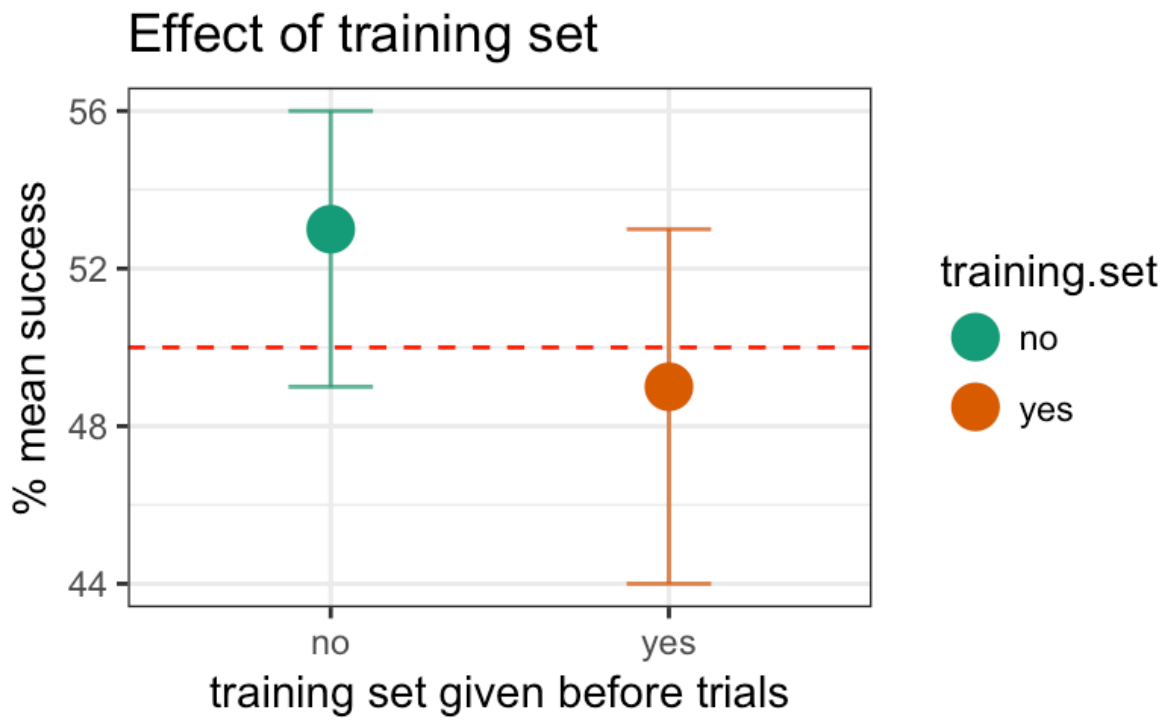



*Figure 77: Plot of training set effect on mean success rate of the group. Either those given a training set: "yes", or not: "no".*

The sample size is extremely small and unlikely to show a difference in the means between the two groups, except in very large effect sizes.

For the success rate, the direct measure of the ability to detect whether there was a difference between the samples in a pair, the null hypothesis (there was no difference between the trained or untrained group) was not rejected in an paired t-test (p-value = 0.3472).

Because there was a long pause between the training set and starting the trials, if there was a training set effect but one masked by habituation of a key olfactory receptor, then one would expect the first sample in each set to be more likely to be accurate than

those which followed. An exact binomial test of the results of the first comparison for each volunteer who had a training test and those for every trial after the first for those who undertook multiple sessions, shows a success rate of 63% in 19 trials, not significantly different to that of random chance (p-value = 0.3593)

**Effect of Professional Experience on Success Rate**

It might be expected that professional perfumers with significant experience in identifying and distinguishing between odours as well as enhanced odour memories, would have a greater success rate than that of untrained smellers. The subset by experience is shown in Figure 78.

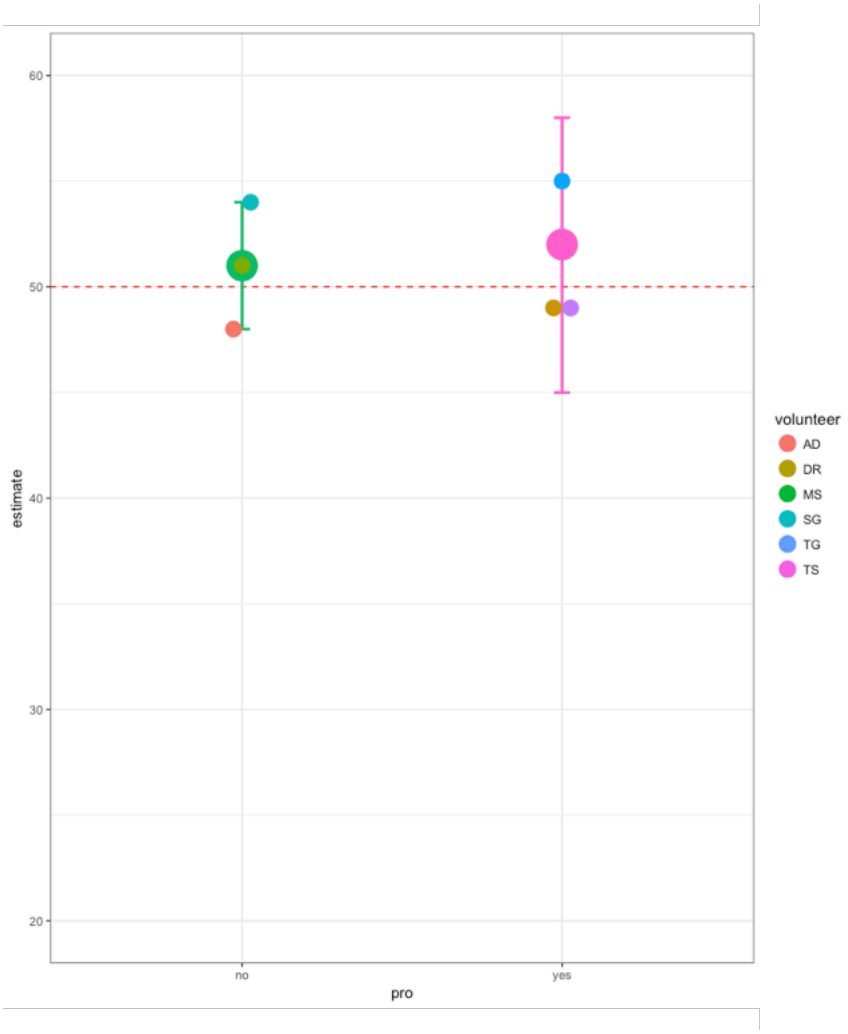



*Figure 78: Plot of experience effect on sample success. Pink and green dots and error bars are mean success and 95% binomial confidence intervals for non-professional and professional smellers. Smaller dots are individual scores per volunteer. There does not seem to be a significant difference between the groups.*

A two-sample t-test demonstrated the means of the professional and "amateur" groups are identical (p=1.0), there is no difference in their ability to determine whether a pair is made up of samples of the same deuteration status by odour.

**Impact of Pairwise Comparisons on Success**

If there were short-lived competitive inhibition of activation of a key OR within the presumed "suite" of receptors activated by the odorants, one might expect the order of presentation of the odorants to make a difference to the task success rate. If the inhibition lasted longer than approximately 30 seconds (the approximate time between pair exposure

due to re-randomisation) then there would be a hold over effect from one trial to the next, which I have not examined. The grouped scores for each pairwise comparison are shown in Figure 79.

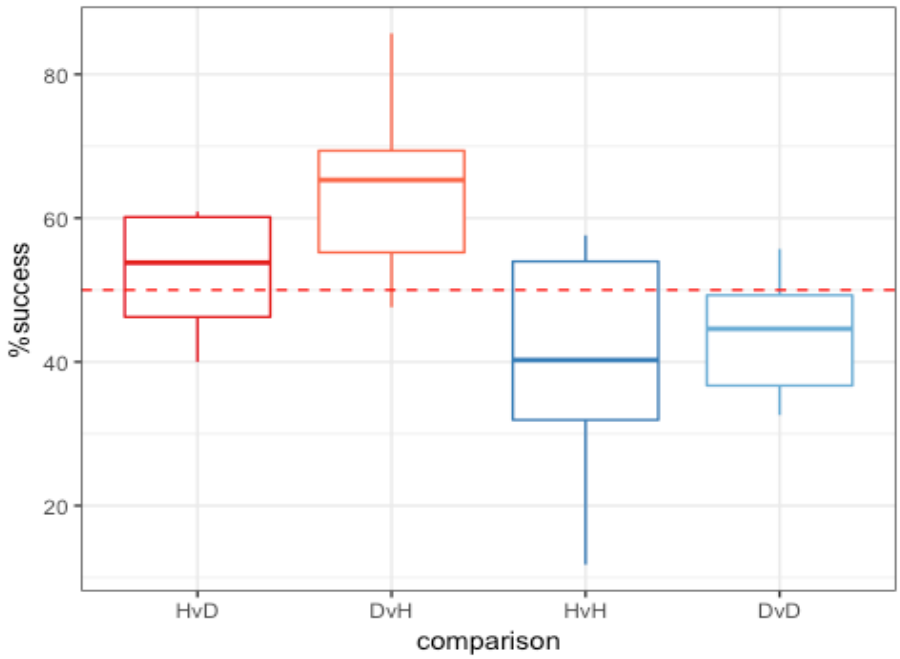



*Figure 79: Comparison of volunteer success rate by pair comparison. There is an apparent difference between the comparisons of pairs of different odorant samples, especially DvH, and those which are the same.*

Because there were multiple observations on the same individuals and assuming a normal distribution, I performed a mixed-effects ANOVA to determine whether any of the groups were significantly different in comparison to the others.

| | Degrees of freedom | Sum sq | Mean Sq | Value | Pr(>F) |
|---|---|---|---|---|---|
| comparison | 3 | 2167 | 722.2 | 5.3015 | 0.0154* |
| Residuals | 20 | 3269 | 163.5 | | |

*Table 12: Analysis of variance results demonstrating a significant variance in at least one of the between-group comparisons at p=0.05*

There is a significant difference in the comparisons between at least one of the groups.

As no intergroup analysis was specified before the experiments were undertaken, I used a post-hoc test: the Tukey HSV (Honestly Significant Variance) with a 95% confidence interval.

| COMPARISON | DIFFERENCE | LOWER BOUND OF 95% CI | UPPER BOUND OF 95% CI | P VALUE |
|---|---|---|---|---|
| **DVH-HVD** | 12.0 | –8.7 | 32.7 | 0.39 |
| **HVH-HVD** | –12.8 | –33.5 | 7.8 | 0.33 |
| **DVD-HVD** | –8.7 | –29.4 | 12.0 | 0.65 |
| **HVH-DVH** | –24.8 | –45.5 | –4.2 | 0.02 |
| **DVD-DVH** | –20.7 | –41.3 | –0.0 | 0.049 |
| **DVD-HVH** | 4.1 | –16.5 | 24.8 | 0.94 |

*Table 13: Tukey HSV comparison table, comparisons a significant difference between them, with p>0.05 are highlighted.*

There is a difference in between the group comparisons which arises from the difference in success between the success in recognising DvH and the lower rate for "same" comparisons, "HvH" and "DvD". This can be explained by the bias in the "yes rate", discussed below, but the question remains why this is not the case for the HvD comparison.

**'Yes' rate**

In the analysis of the results I found evidence of a response bias: most volunteers had a low overall rate of identifying the sample pair as similar, i.e. answering "yes" to the experimental question "Do these samples smell the same?". One would expect this to be at around 50% if there were no detectable difference between the samples but also at chance level if there *were* a detectable difference as the overall distribution between the comparisons was still 50/50.

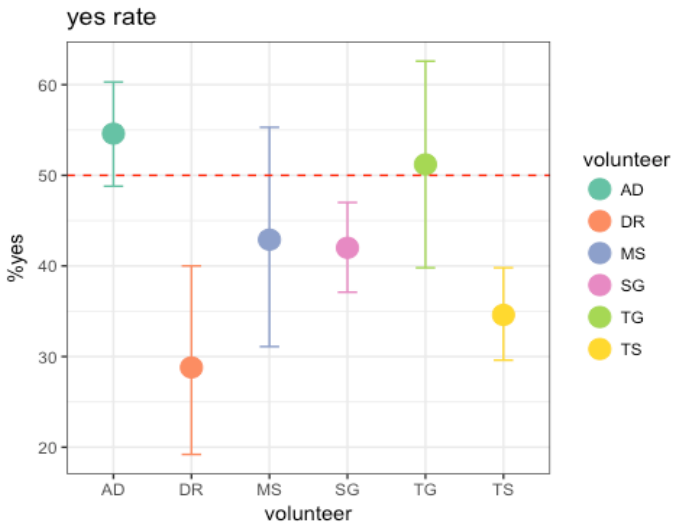



*Figure 80 The overall percentage that a sample pair was said to be "the same", showing that for most volunteers there was a bias towards perceiving a difference between the samples, whether or not they were different (significant bias in half of the volunteers).*

| ID | YES | TRIALS | % | LOWER BOUND OF 95% CI | UPPER BOUND OF 95% CI | P VALUE | TRAINING SET GIVEN? | PROFESSIONAL SMELLER? |
|---|---|---|---|---|---|---|---|---|
| DR | 23 | 80 | 28.8 | 19 | 40 | 0.00 | YES | YES |
| TG | 41 | 80 | 51.2 | 40 | 63 | 0.91 | NO | YES |
| TS | 121 | 350 | 34.6 | 30 | 40 | 0.00 | YES | YES |
| AD | 175 | 320 | 54.6 | 49 | 60 | 0.10 | NO | NO |
| SG | 168 | 399 | 42.0 | 37 | 47 | 0.00 | NO | NO |
| MS | 30 | 70 | 42.9 | 31 | 55 | 0.28 | YES | NO |

*Table 14: 'Yes'-rate for each volunteer with binomial significance calculated with Pearson-Clopper exact method. YES is the number of replies saying there was a detectable difference between the samples, TRIALS is the total number of trials, % is the percentage result.*

The results are graphed in Figure 80 and expressed in Table 15, with a one-sample t-test there was no significant bias in the group as a whole ($p = 0.118$), there are certainly three volunteers (DR, SG, TS) who are more likely to underestimate the likelihood of a sample pair being the same.

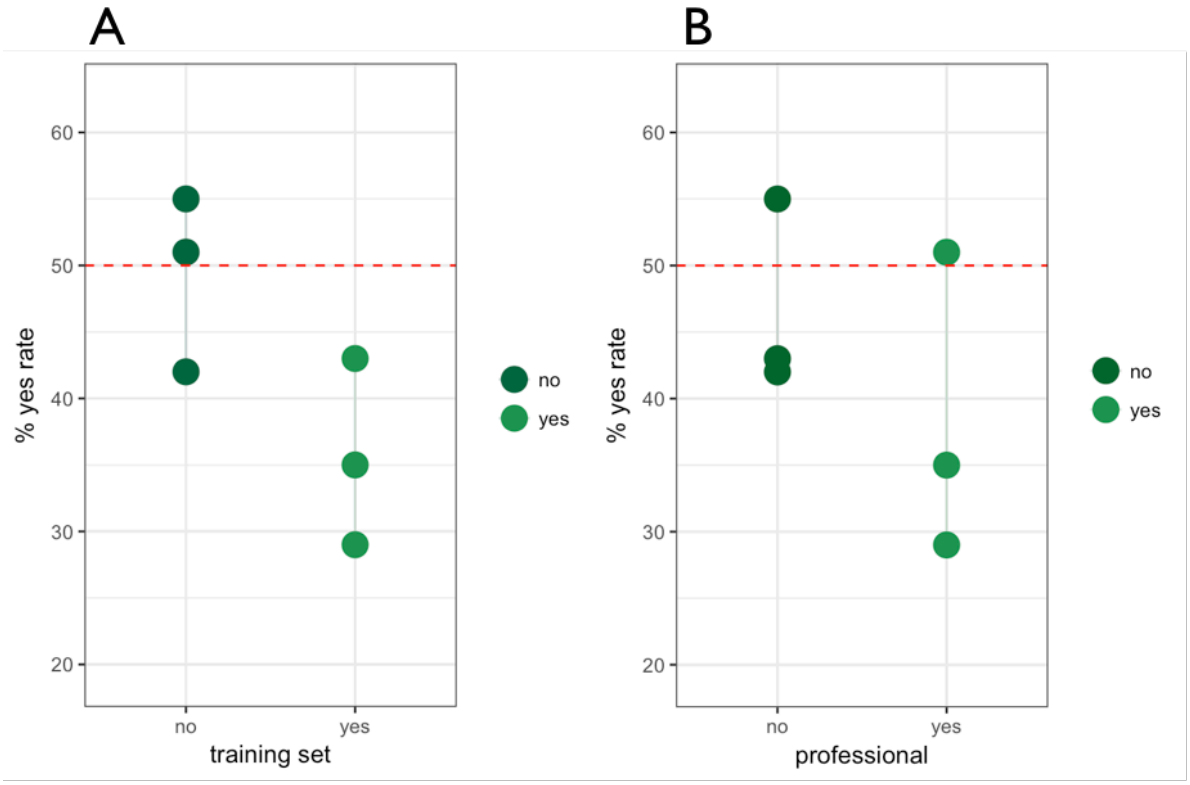



*Figure 81: Plots of training set and professional experience effect on the 'Yes'rate. Plot A is the comparison of the yes rate in those which received a training set prior to the blinded experiments. Plot B is the comparison of false response rate in between professional and naïve smellers.*

Effect of Training Set on Yes Rate

The training set had no effect on the success of the candidates at distinguishing between the two samples, but did it affect the bias against declaring the two samples the same? The 'yes' rate (the number of times a volunteer declared a sample pair to be "the same" for the trained and untrained group is shown in Figure 81. There appears to be an effect on the 'yes' rate, consistent with some of the literature in visual psychophysics that learning can increase the false response rate (Wenger et al. 2008).

Testing the null hypothesis that there is no difference between the groups at a 95% CI, I performed a two-sample t-test in R and could not reject the null (p-value = 0.9645):

The bias against finding the two samples the same was significantly *increased* by exposure to a training set before the blinded trials were begun, as measured by one-tailed

t-test with a 95% CI. I would have expected that the training set would increase the confidence to identify the similarity between samples and to therefore decrease the bias. This is a powerful indication that the summation of olfactory inputs is a difficult one, with what is likely to be many complex sensory qualia needing to be summarised into one 'yes-no' answer.

Effect of Professional Experience on Yes Rate

Professional experience might be expected to make a volunteer more confident in their olfactory ability and therefore more confident in pronouncing a sample pair "the same", but this was not the case, although there is also evidence that learning can increase the false positive response bias (P. R. Jones et al. 2015). A two-sample t-test failed to show any correlation between the professional status and yes rate (p-value = 0.8228).

Caution must be exercised in drawing causative inferences from these results, the sample size is very small and the overlap between perfumer status and training set is comparatively large (two out of three perfumers had a training set). It does, however, show that there is a surprising bias against reporting the main finding of the experiment, namely that there is no difference in the odour of the deuterated and hydrogenated samples.

**Individual analysis**

Although there was no significant finding within the groups, I analysed the individual results to examine whether there were any factors which could be identified on a per-volunteer basis. Was the overall inability to detect the difference between the samples because of other factors, such as inhibition or habituation? Additionally, some of the subjects undertook other versions of the experiments, none of which were successful in demonstrating any difference in odour quality but I present these to exclude them as methods for further work.

Subject TG

Subject TG was a perfumer with over 20 years of experience in the industry. He achieved a 36/40 (normosmic) on the UPSIT and 6 on the SNOT-22 (minimal nasal symptoms). He reported a benzyl-benzoate specific anosmia he had noticed in his own work (unlikely to affect his ability to detect acetophenone).

He undertook one session of 80 samples in one sitting with breaks. This was preceded by a training session of two sample pairs identified and presented in order, and then reversed.

| |
|---|
| percentage success: 47.5%<br><br>'yes' rate: 51.2% |

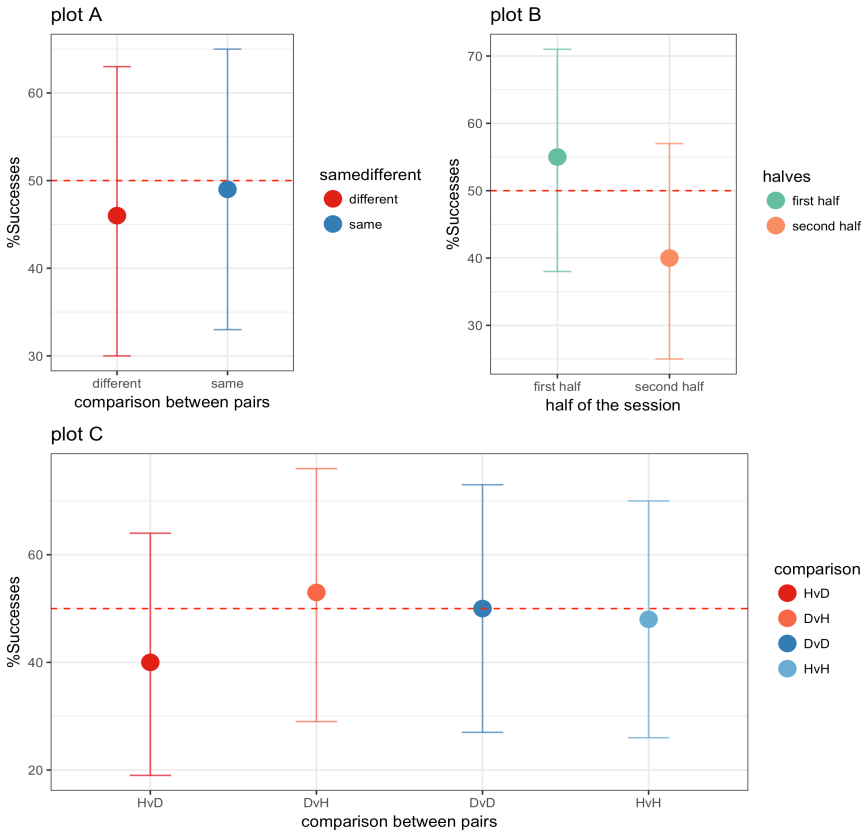



*Figure 82: Plots of %success between (A) same (e.g. a deuterated followed by deuterated sample) and different pairs, (B) first and second half of the test and (C) pairwise comparisons for TG*

In Figure 82, plot A shows that TG demonstrated no significant difference in the ability to identify the pairs if they consist of the same or different molecules. This is broken down by specific comparison (deuterated and then undeuterated is DvH, for instance) in plot C. Here there is a slight (non-significant) decrease in the ability to identify a non-deuterated sample followed by a deuterated one.

Plot B is an attempt to show whether any learning took place, but if anything, the volunteer was worse at the task in the latter 40 samples than he was in the first half of the experiment (again this is a non-significant finding). This could be evidence of habituation, which was a recognised risk of using acetophenone.

Identification Task

In addition to the standard trial procedure TG attempted to identify each sample individually, without comparison to the partner sample. He was no better at determining which sample was deuterated and which was not, with an overall success rate of 43.2%, the breakdown by sample and session position being shown in Figure 83. I abandoned this as a trial methodology in later trials.

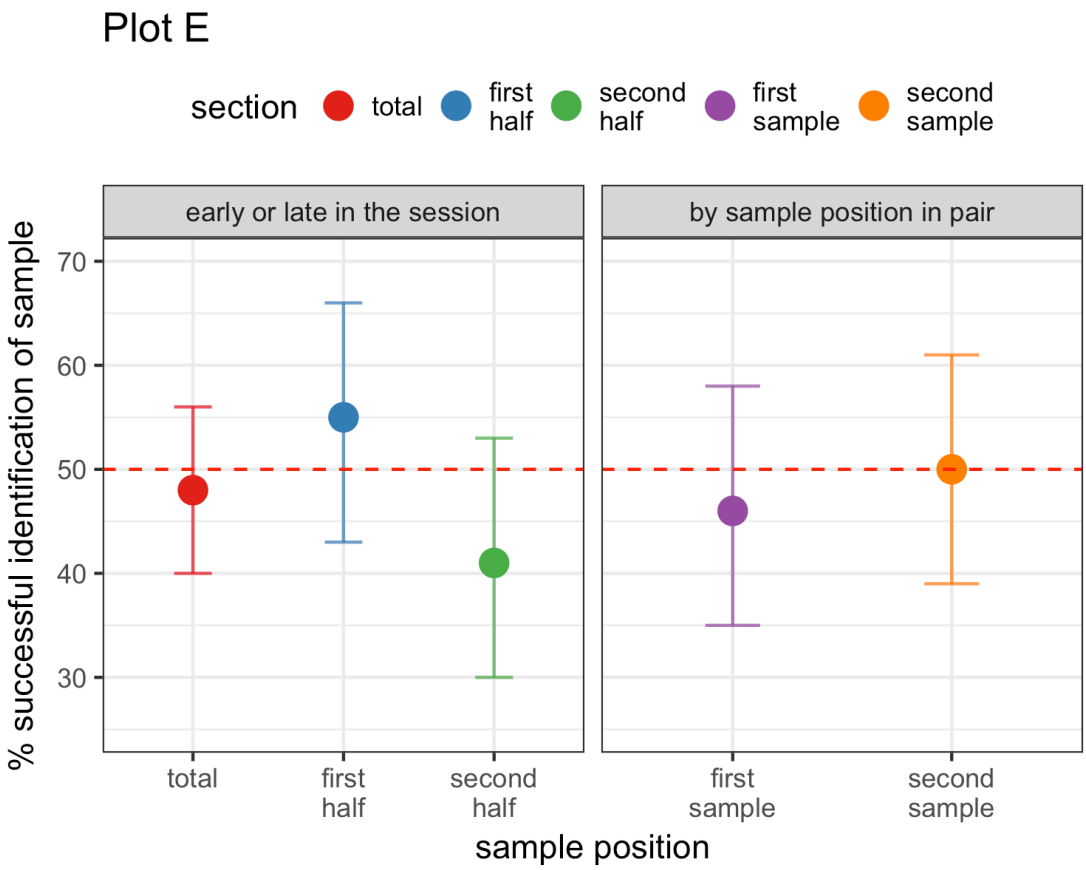



*Figure 83: Plot E - plot of the successful identification of sample type (not comparison) for each sample for first and second halves of the experimental session. The subject was unable to significantly identify the sample type in either half of the session, or either position in the pair.*

Subject TS

Subject TS has been a perfumer for over 25 years. He scored a normosmic 37/40 on the UPSIT test and a SNOT-22 score of 19, indicating mild disease (median normal score is 7)

(Hopkins et al. 2009). He undertook multiple sessions of the experiment, over two days with several different batches of the odorants.

percentage success: 48.3%

'yes' rate: 34.6%

| session | "yes rate" |
|---|---|
| 1 | 30% |
| 2 | 52% |
| 3 | 57% |
| 4 | 45% |

*Table 15: Tabular overall results for subject TS*

In Figure 84, plot A there is a non-significant variation (p=0.187) in the ability to distinguish between similar and different pairs of odorants. This can be explained by the volunteer's low yes rate. He showed a significant reluctance to identify the pairs as being similar (as seen in Figure 83), a bias which, in a random selection of samples will give the skew shown above.

This is recapitulated by the individual pairwise comparisons in plot B, which shows very little variation depending on the sample type presented first in the pair, although once again the DvH comparison is the most likely to be correctly identified. It might be expected that the bias against identifying the samples as "the same" would change over the sessions as the results were reported back after each session, but the 'yes' rate (the green bars in plot C) decreased over the sessions with no apparent impact in the success rate. A Chi-squared test for trend in proportions showed a significant linear downward trend in the 'yes' rate with repeated testing (p = 0.025). There was a significant improvement in the successful identification of the samples from the first session, but not to above the rate expected by chance.

Plot D does show an increasing split between the "same" and "different" pair success rates over the sessions, although this is a reflection of the overall decreased 'yes' rate. Once

again, the DvH comparison is the most likely to be correctly identified as begin different, in all sessions apart from the first.

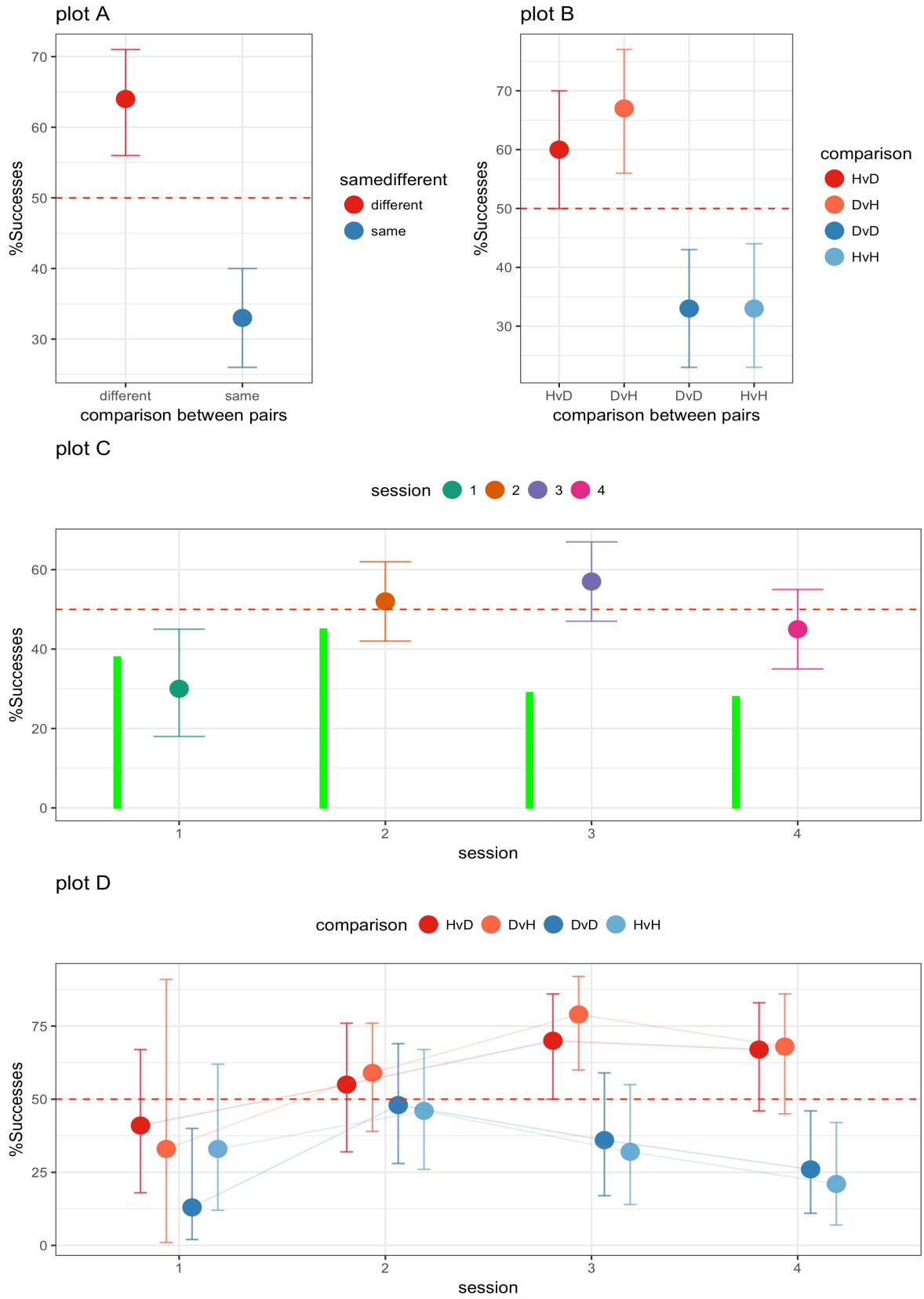



*Figure 84: Plots of %success between (A) same and different pairs, (B) pairwise comparisons, (C) score over sessions, green bars represent the yes rate in each session, (D) pairwise comparisons per session for volunteer TS*

Subject DR

DR has been a perfumer for over 15 years, who has a self-identified (and not experimentally confirmed) partial benzyl salicylate anosmia. He reported a below-normal SNOT-22 score of 5 and identified 36 odours out of 40 on the UPSIT test.

He undertook the trial at one sitting. The samples were presented without a training set and he was only allowed one sniff at each.

| |
|---|
| percentage success: 52.5% |
| 'yes' rate: 28.8% |

*Table 16: Tabular overall results for subject DR*

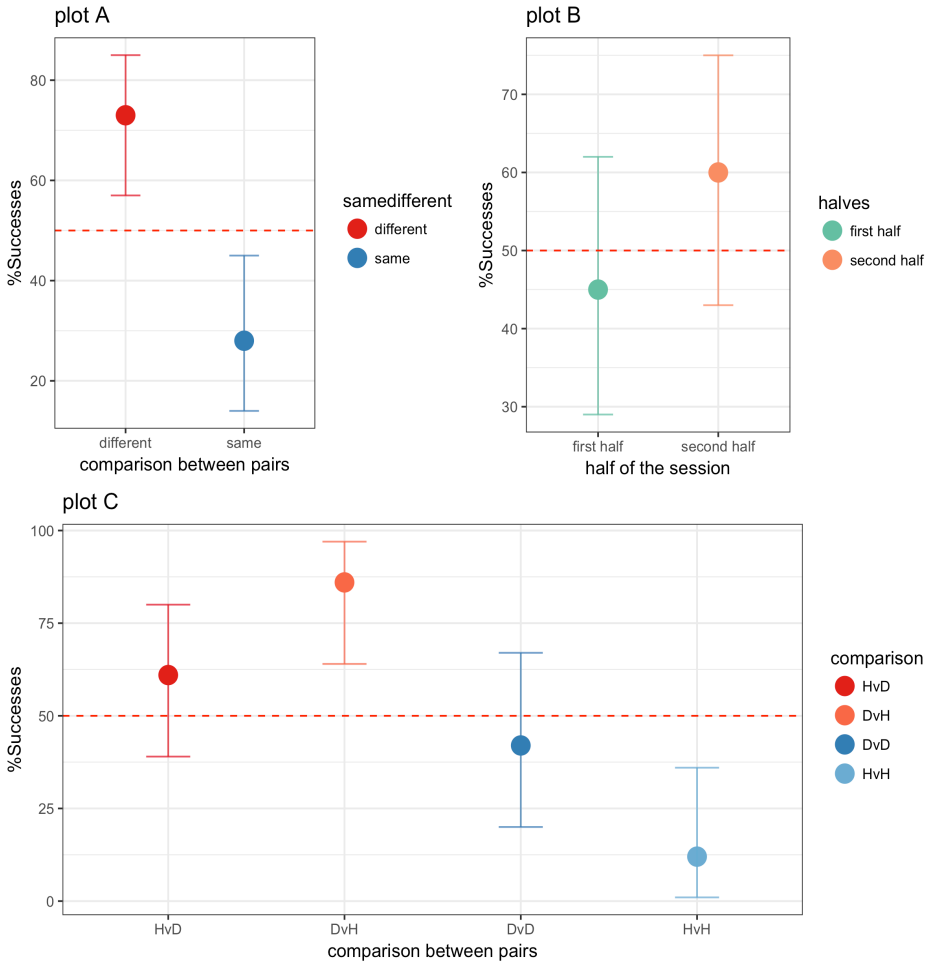



*Figure 85: Plots of % success between (A) same and different pairs, (B) first and second half of the test and (C) pairwise comparisons for DR*

Again, he had a very low "yes rate" (despite having a training set before he started), being much more likely to report a difference between the samples. This skewed the results as expected in plot A, rendering a difference between the two which was not significant on the t-test (p=0.312).

In plot B, although not significant (p = 0.099), there is the appearance of improvement between the first and second halves of the session. Once again, the pairs presented with the deuterated sample first were much more likely to be correctly identified.

Subject MS

MS was an untrained smeller, although interested in smell. He was a physicist. Unfortunately, he was not able to complete the SNOT-22 and UPSIT tests, although my clinical opinion was of no significant nasal disease and a normal sense of smell, because of our many conversations on the topic and multiple opportunities of smelling various molecules together. These data were excluded from the published version of these results because of the lack of objective evidence of his normal olfactory function. He undertook the trial at one sitting.

| |
|---|
| percentage success: 45.7% |
| ‘yes’ rate: 42.9% |

*Table 17: Tabular overall results for subject MS*

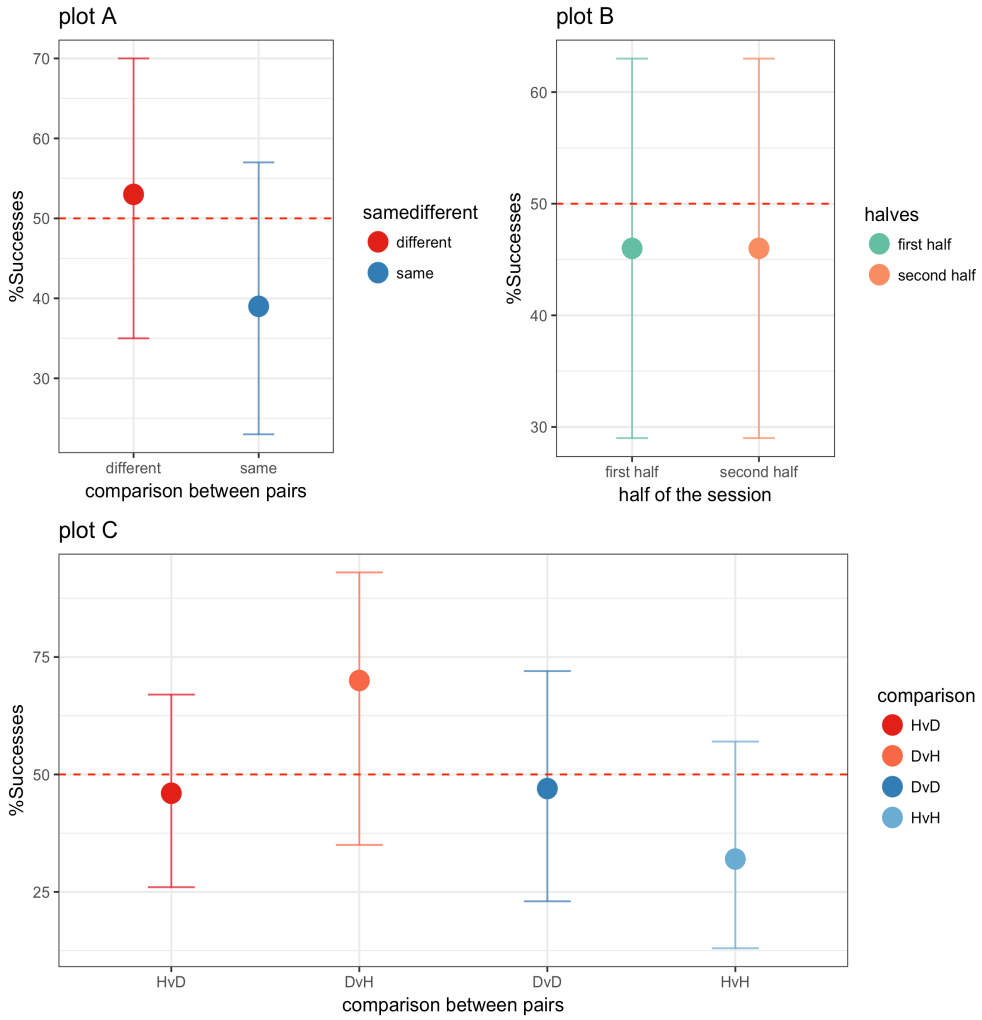



*Figure 86: Plots of % success between (A) same and different pairs, (B) first and second half of the test and (C) pairwise comparisons for MS*

Once again the subject demonstrated no consistent ability in to identify the same or different sample pairs. He showed the most consistency across the session (Figure 86, plot B), with no improvement or decrement in his success at picking the right answer. Like all but one of the volunteers, he was most successful at identifying the DvH pairs as being different.

Further Smelling Experiments:

MS undertook further tests to attempt control for habituation or inhibition by leaving a significant amount of time between trials, usually several hours. Unfortunately, he was taken ill before he could complete this experiment.

I was concerned that the samples would lose their strength over this period as there is a drop off in success rate in these data, but this appears to have been a promising avenue of investigation. The success rate is not significantly greater than chance on an exact binomial test (p-value = 0.21) and I only obtained these results after the other trials had been completed.

As this experimental method was the only one which lasted over multiple days, it was the only method which would have been sensitive to attrition.

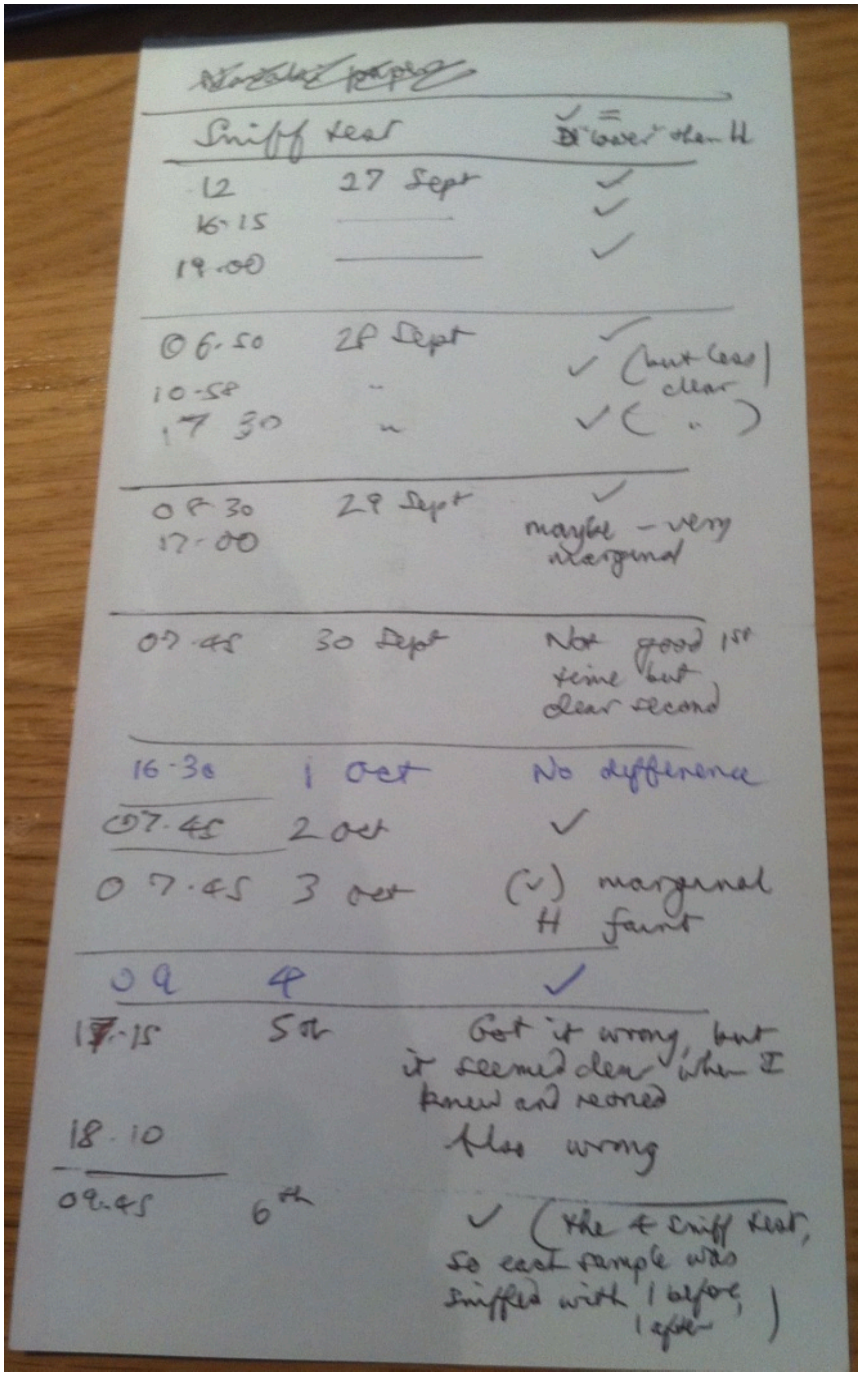



*Figure 87: Twice daily trials to attempt to control habituation/inhibition experiment: MS's hand written results.*

Subject AD

AD was a post-graduate physics student and achieved 36/40 on the UPSIT and 15 on the SNOT-22 (above median but still mild disease, the highest-scoring symptoms were non-specific for nasal disease), she was a non-perfumer but had experience of odour comparison as she worked in a specialist whisky shop and had a research interest in smell.

She undertook the experiment over multiple days, one session per day.

percentage success: 52.3%

'yes' rate: 54.6%

| session | yesrate |
|---|---|
| 1 | 38.9% |
| 2 | 62.3% |
| 3 | 48.1% |
| 4 | 67.4% |
| 5 | 62.7% |
| 6 | 60% |

*Table 18: Tabular overall results for subject AD*

This volunteer is anomalous in several ways: she did not have the same overall low 'yes' rate as most of the others, although she did during the first session (Chi-sq test for proportions p=0.008). She also had a significant difference (plot A in Figure 88) between the comparisons of same and different pairs on t-testing (p-value for the comparison between same and different pairs: 0.045). Related to this was her inversion of the usual ability to detect a difference of the pair when presented with the deuterated sample first (plot B in Figure87).

Plot D shows no clear separation of the abilities to distinguish between the samples although there are tantalizing hints of improvement over the days (no linear trend is shown on the Chi-squared test for trend in proportions, p-value = 0.5502).

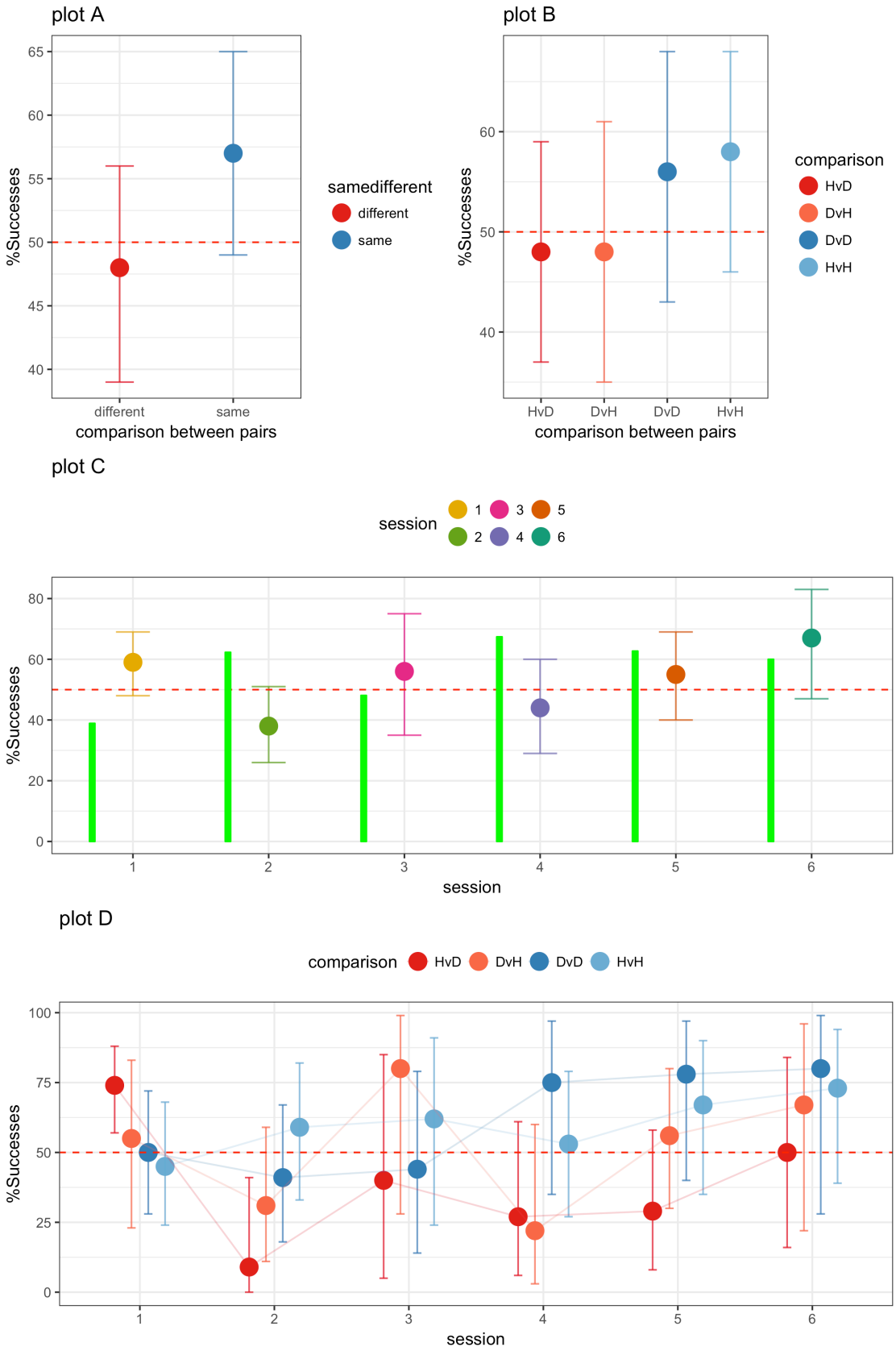



*Figure 88: Plots of %success between (A) same and different pairs, (B) pairwise comparisons, (C) score over sessions, green bars indicate 'yes' rate per session (D) pairwise comparisons per session for volunteer AD*

Subject SG

I am volunteer SG. I scored a 39/40 on the first attempt at an UPSIT, undertaken about a year before this experiment, but not repeated during this experiment because the training effect would be likely to give a skewed answer. I had mild allergic rhinitis which was treated with sublingual immunotherapy several years before this trial and at the time of the experiment had a SNOT-22 score of 1. For my trials, the experimenter was my post-graduate student colleague, although the experimental structure and double blinding technique meant that I would have been able to perform the tests on my own. I had a few years of experience in odour comparison due to my research interest.

percentage success: 54.5%

'yes' rate: 42%

| session | yesrate |
|---|---|
| 1 | 45% |
| 2 | 20% |
| 3 | 30% |
| 4 | 42.6% |
| 5 | 46.7% |
| 6 | 48% |
| 7 | 38% |
| 8 | 50% |
| 9 | 30% |

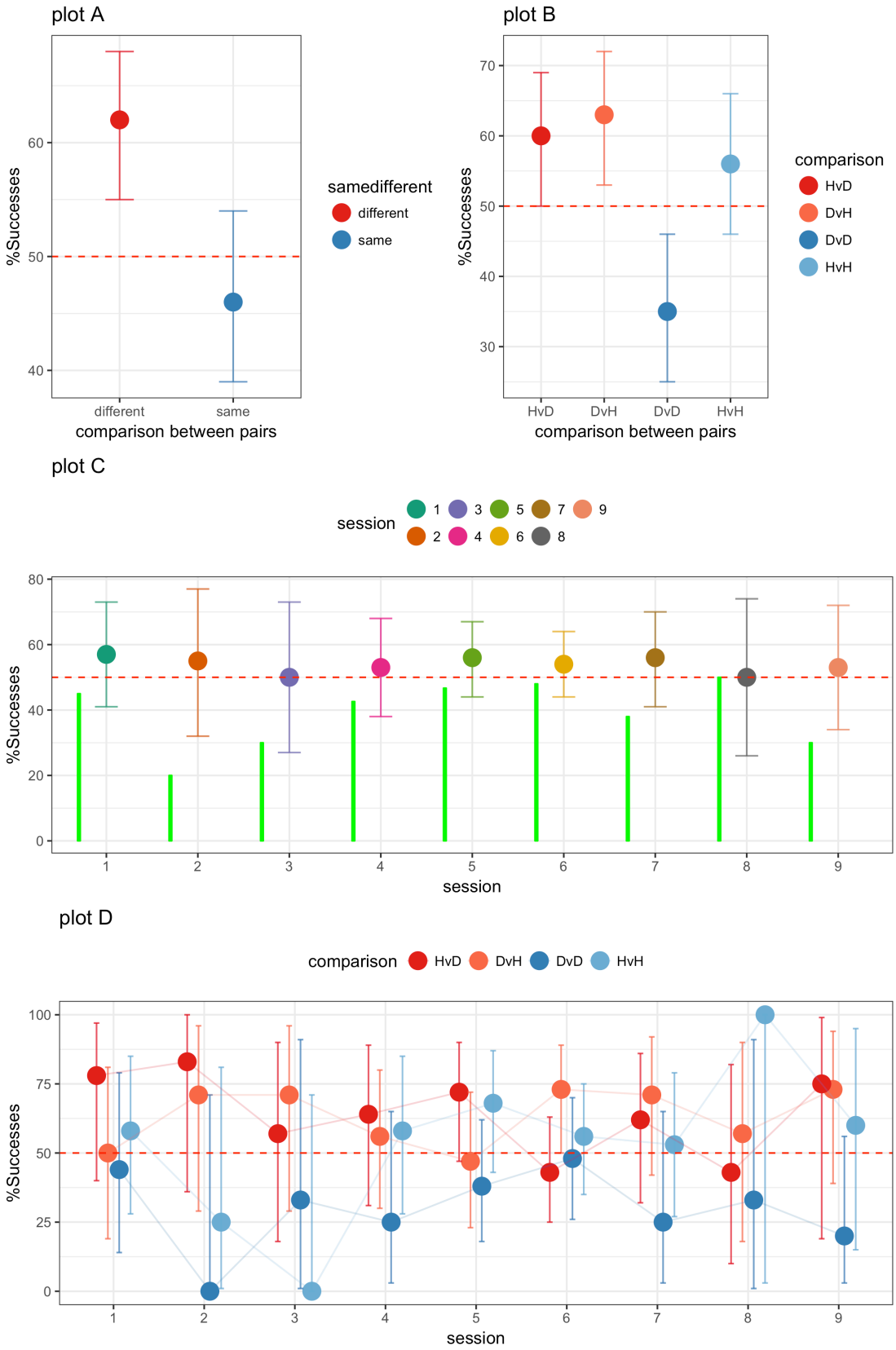



*Figure 89: Plots of % success between (A) same and different pairs, (B) pairwise comparisons, (C) score over sessions, green bars represent the 'ye's rate per session (D) pairwise comparisons per session for volunteer SG*

Even though SG was perhaps the closest in showing a significant ability to detect the difference between the samples, there was no significant difference in success of the comparison between same and different pairs: (p=0.104) in plot A, Figure 89. He also showed the skew towards the DvH identification success with Chi-squared test for trend in proportions p=0.001.

There was no learning effect, i.e. improvement of success over the sessions using a Chi-squared test for trend in proportions: (p=0.798), neither was there significant difference in the 'yes' rate over sessions (p=0.263) and therefore no trend could be expected in this either.

An ANOVA assessment of the correlation between the 'yes' rate and success indicates no impact of the 'yes' rate on the success of detection (p=0.905).

**Summary of Acetophenone Results**

In a controlled environment, I was unable to demonstrate that deuteration alters the odour of acetophenone to humans with double-blind testing as shown in Figure 90.

This is true in all volunteers both those with and without experience in olfactory assessment. There is a marked bias in finding the samples to have the same smell in most volunteers, but this did not affect the findings. Most volunteers were successful in distinguishing between the samples as long as they were presented with the deuterated sample first (DvH).

This experiment did not support the hypothesis that altering the vibrational characteristics of a molecule altered its smell.

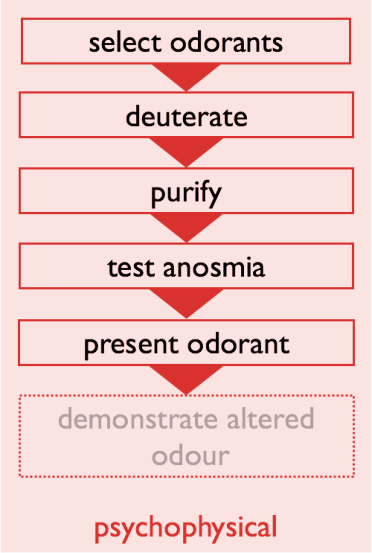



*Figure 90: Failure to show an alteration in the odour of isotopes*

## Exaltone

Musks represented a very interesting probe molecule pair as discussed. The experimental method required some slight modification but the underlying objective remained:7 to demonstrate an altered odour in a deuterated version of a molecule.

### Assessment of Partially Deuterated Musks

During the synthesis of the deuterated musk, to correlate the extent of deuteration with alteration in odour character, samples of cyclopentadecanone were deuterated to differing extents, by halting the deuteration reaction prematurely. The normalized GC traces corresponding to each sample are shown in Figure 91. The samples were purified and evaluated by trained observers at Vioryl SA (Gane et al. 2013). The deuterated character was undetectable only in the sample with the lowest percentage of deuteration [sample 1] and present in the others, suggesting that less than 50% deuteration [14 deuteriums] is insufficient to change odour character. The wide range of deuteration present in all samples prevented a more accurate estimate on initial testing but further elucidation of this phenomenon is planned.

These results are subjective and were not controlled or double-blind. Further work is required to demonstrate this fully.

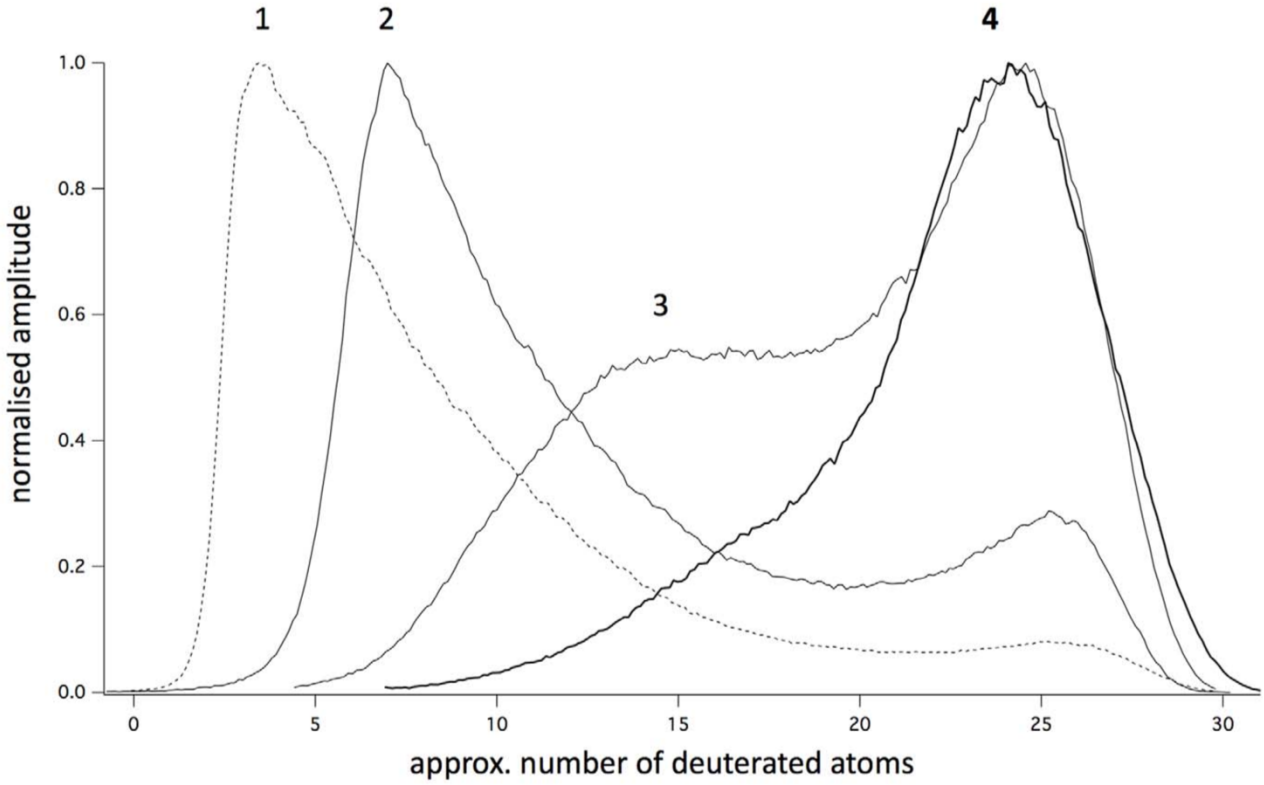



*Figure 91: GC elution profiles of partially deuterated cyclopentadecanone samples. The GC traces have been normalised to a peak amplitude of 1 and are shown plotted against approximate deuteration number. The deuteration scale is taken from mass spectra taken at different times and differs slightly between one sample and the next because of slight differences in the GC elution times. The abscissa should therefore be taken as approximate, i.e+/22 deuterated atoms. The sample depicted in trace 1 had no discernible burnt odour. Data from Vioryl SA*

**Results for All Volunteers**

| VOLUNTEER | AGE | GENDER |
|---|---|---|
| KM | 28 | F |
| JB | 32 | M |
| LT | 59 | M |
| AM | 32 | M |
| CS | 31 | F |
| MG | 34 | F |
| AD | 27 | F |
| NH | 31 | M |
| VC | 26 | F |
| CC | 28 | F |
| KF | 32 | F |

*Table 19: Table of demographics for the volunteers*

The demographics of the volunteers are presented in Table 19 and the summarised results are given in Table 20 below.

| VOLUNTEER | SUCCESS | TRIALS | ESTIMATE% | LOWER BOUND OF 95% CI | UPPER BOUND OF 95% CI | PVALUE |
|---|---|---|---|---|---|---|
| KM | 17 | 17 | 100 | 80 | 100 | **0.00** |
| JB | 12 | 12 | 100 | 74 | 100 | **0.00** |
| LT | 12 | 12 | 100 | 74 | 100 | **0.00** |
| AM | 12 | 13 | 92 | 64 | 100 | **0.00** |
| CS | 11 | 12 | 92 | 62 | 100 | **0.01** |
| MG | 9 | 10 | 90 | 55 | 100 | **0.02** |
| AD | 10 | 12 | 83 | 52 | 98 | **0.04** |
| NH | 10 | 12 | 83 | 52 | 98 | **0.04** |
| VC | 9 | 12 | 75 | 43 | 95 | **0.15** |
| CC | 9 | 12 | 75 | 43 | 95 | **0.15** |
| KF | **6** | **8** | **75** | **35** | **97** | **0.29** |

*Table 20: Table of results with binomial significance calculated with Pearson-Clopper exact method.*

The findings confirmed the initial subjective assessment from the partial deuteration experiment - almost all the subjects could identify the deuterated musk in the pair with a CI of 95% (eight subjects). Three subjects were not able to distinguish the sample with a CI of 95%, but these all had short trials for several reasons. At a higher significance requirement,

CI = 99%, around one half of the subjects detected a difference between the samples, with the true mean falling above that of chance.

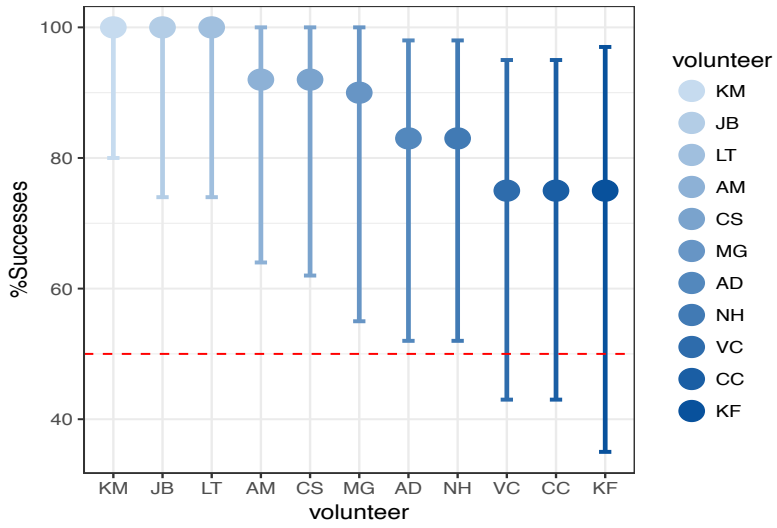



*Figure 92 Individual results by volunteer with binomial significance calculated with Pearson-Clopper exact method combined result for all trials. (plot of the results of Table 20)*

To assess the significance of the group's success I performed a single-sided one sample t-test, with the null hypothesis being that the ability to correctly identify the deuterated sample in a pair is not greater than chance (null probability = 50%).

A one-tailed, one sample t-test of the group shows that, as a whole, the group was significantly better than random chance would predict at a CI = 99% at a p-value of p-value = 1.102e-07 and we can therefore reject the null. There was a perceptible difference in the odour of the two samples.

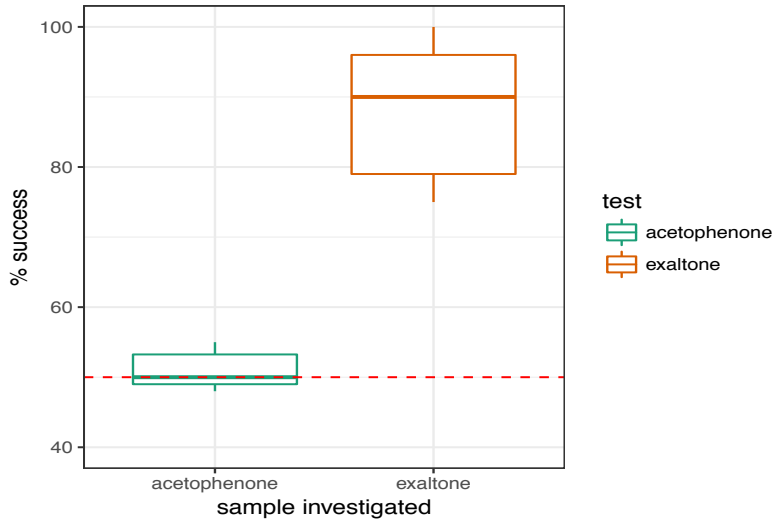



*Figure 93: Boxplot of acetophenone vs Exaltone results, showing significant difference between the results*

**Summary of Exaltone Results**

For this experiment, the null can be rejected: untrained humans can detect a difference between deuterated and non-deuterated musks by odour alone. We successfully demonstrated that to humans, deuterated Exaltone has a markedly different character to that of its hydrogenated counterpart and the first part of the project is successful.

## Chapter summary

This chapter has demonstrated that deuteration of some molecules (Exaltone and perhaps other musks) alters the odour for humans, whereas for others (acetophenone and toluene) it does not.

Expertise is perfumery or having a training set given before the trials, made no difference in the detection of the deuterated acetophenone. The odour change for Exaltone was so obvious that no further training or expertise was required.

The question remains as to whether this is a receptor-level event and if so, which receptor was responsible for the detection of Exaltone. Finding the musk receptor(s) is the subject of the next chapter.

# Chapter Four: Results for the Genetics of Musk Anosmia

## Chapter synopsis

The identification of a musk anosmic family allowed us to use modern genetic techniques to identify a musk receptor, with the aim of expressing that in a heterologous expression system and examining its function under controlled conditions.

Three families were identified, phenotyped and genotyped.

## Phenotype

Three Greek families were recruited as discussed in the Methods chapter, and the results of the phenotyping are summarised in Table 21:

| FAMILY | VOLUNTEER | RELATION | GENDER | SNIFFIN' STICKS SCORE | SMOKER | DNA TRANCHE COLLECTED |
|---|---|---|---|---|---|---|
| **1** | 01.01 | index | M | 10 | n | 1 |
| | 01/02 | father | M | 10 | n | 1 |
| | 01/03 | mother | F | 9 | n | 2 |
| | 01/04 | nephew (sister's son) | M | 11 | NA | 1 |
| | 01/05 | wife | F | 12 | NA | 1 |
| | 01/06 | daughter | F | 10 | n | 1 |
| | 01/07 | sister | F | NA | y | 2 |
| **2** | 02/01 | index | F | 12 | y | 1 |
| | 02/02 | mother | F | 11 | n | 1 |
| | 02/03 | father | M | 10 | y | 1 |
| | 02/04 | brother | M | 12 | y | 1 |
| **3** | 03/01 | index | F | NA | n | 2 |
| | 03/02 | sister | F | NA | n | 2 |
| | 03/03 | mother | F | NA | NA | 2 |
| | 03/04 | father | M | NA | NA | 2 |
| | 03/05 | maternal aunt | F | NA | NA | NA |
| | 03/06 | niece | F | NA | NA | NA |
| | 03/07 | niece | F | NA | NA | NA |

*Table 21 Demographics for three Greek families recruited with baseline olfactory function and DNA tranche collected, NA: not available.*

Demographics for the three families are presented in Table 21. The results from the initial 'yes/no' testing for anosmia and the triangle testing with 50% Galaxolide are presented in Table 22. Table 23 presents the results for threshold testing for Galaxolide and Exaltone.

| VOLUNTEER | GALAXOLIDE | HABANOLIDE | MUSK KETONE |
|---|---|---|---|
| **01/01** | anosmic | anosmic | anosmic |
| **01/02** | osmic | anosmic | anosmic |
| **01/03** | osmic | anosmic | anosmic |
| **01/04** | osmic | osmic | osmic |
| **01/05** | osmic | osmic | osmic |
| **01/06** | osmic | osmic | osmic |
| **02/01** | anosmic | anosmic | NA |
| **02/02** | osmic | osmic | NA |
| **02/03** | anosmic | anosmic | NA |
| **02/04** | osmic | osmic | NA |
| **03/03** | anosmic* | NA | NA |
| **03/04** | osmic* | NA | NA |
| **03/05** | anosmic* | NA | NA |
| **03/06** | osmic* | NA | NA |
| **03/07** | osmic* | NA | NA |

*Table 22: Results for yes/no detection of musks and triangle testing for 50% Galaxolide for three families. Triangle testing results are marked with an asterisk. NA: not available*

| VOLUNTEER | GALAXOLIDE THRESHOLD | GALAXOLIDE DILUTION STEP | GALAXOLIDE RANK | EXALTONE THRESHOLD | EXATONE DILUTION STEP | EXALTONE RANK |
|---|---|---|---|---|---|---|
| **01/01** | 100% | 1 | 7.5 | 0.25% | 8 | 2.5 |
| **01/02** | 2.50% | 5 | 4 | 1.00% | 6 | 5.5 |
| **01/03** | 100% | 1 | 7.5 | 0.25% | 8 | 2.5 |
| **01/07** | 100% | 1 | 7.5 | 10.00% | 3 | 7 |
| **02/01** | 100% | 1 | 7.5 | 2.50% | 5 | 6.5 |
| **02/02** | 1% | 6 | 2.5 | 2.50% | 5 | 6.5 |
| **02/03** | 100% | 1 | 7.5 | 1.00% | 6 | 5.5 |
| **02/04** | 1% | 6 | 2.5 | 0.10% | 9 | 1 |
| **03/01** | 100% | 1 | 7.5 | 0.50% | 7 | 4.5 |
| **03/02** | 0.10% | 9 | 1 | 0.50% | 7 | 4.5 |

*Table 23: Phenotype of three Greek families with results for threshold testing. Showing minimum concentration detected ("threshold"), and the relative rank of this threshold for non-parametric testing.*

**Inheritance Pattern for the musks STVs**

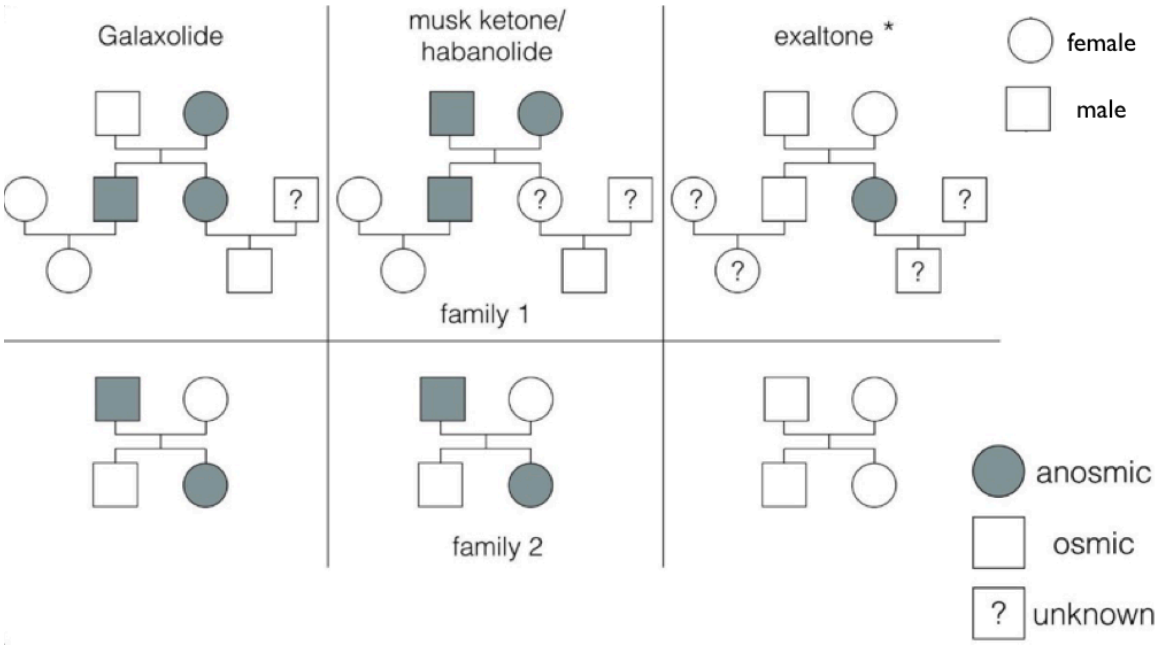



*Figure 94: Genograms for two families for specific anosmias for Galaxolide, musk ketone/Habanolide and Exaltone do not show the same pattern within the families.*

As shown in Figure 94, there are multiple generations in each family with affected members, consistent with a genetic cause.

**Thresholds for Galaxolide and Exaltone® Are Not Correlated**

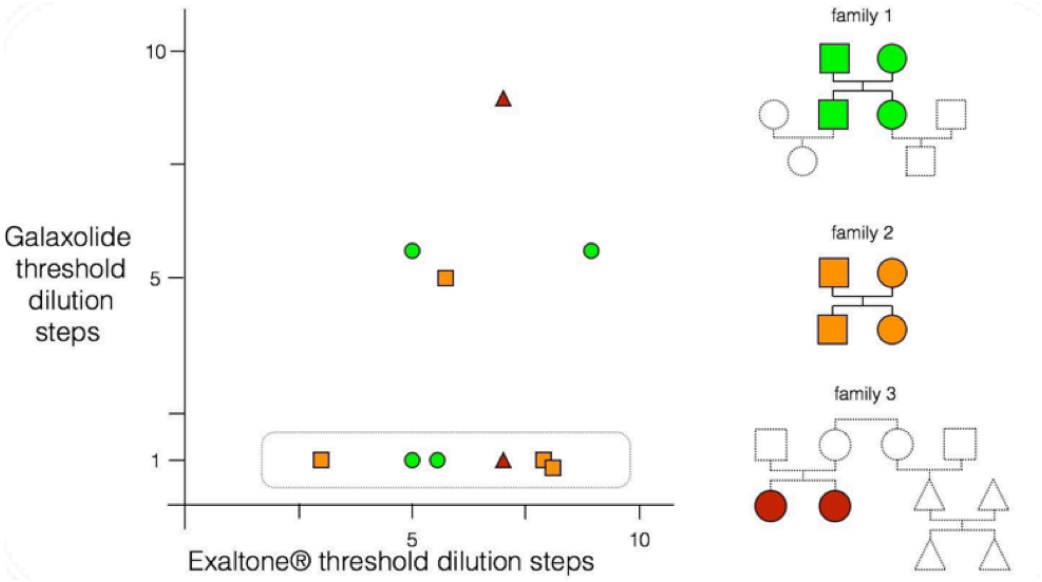



*Figure 95: Perception threshold of Galaxolide does not correlate with that of Exaltone. Grey bar demonstrates the complete Galaxolide anosmics.*

The scatter plot in Figure 95 demonstrates little obvious correlation between the dilution step thresholds for Exaltone® and Galaxolide. Statistical assessment of the relative thresholds is not simple. No assumption of normality in the distribution can be made and additionally there is no guarantee that there is a linear relation between the perceived magnitude of a dilution step and the next. For example, the perceived difference in intensity between 1% and 2.5% dilutions might appear great to a subject whereas the difference between 1% and 0.5% might be undetectable.

Spearman's rank correlation controls for the non-parametric, non-linear possibilities of the correlation between the two variables but does assume a monotonic relationship between the two (i.e. there cannot be, for instance, a U-shaped or sine correlation between them).

Several of the ranks were "tied" between different subjects, for instance all of the completely Galaxolide-anosmic subjects were ranked together. I used the standard practice of averaging the rank between the tied values to correct for this. Unsurprisingly Spearman's rho = 0.145, suggesting a poor correlation between the thresholds for Galaxolide and Exaltone® in our subjects, although the finding does not achieve standard significance with $p > 0.5$ (p-value = 0.69), due to the low number of participants.

**Smoking Does Not Affect Anosmia**

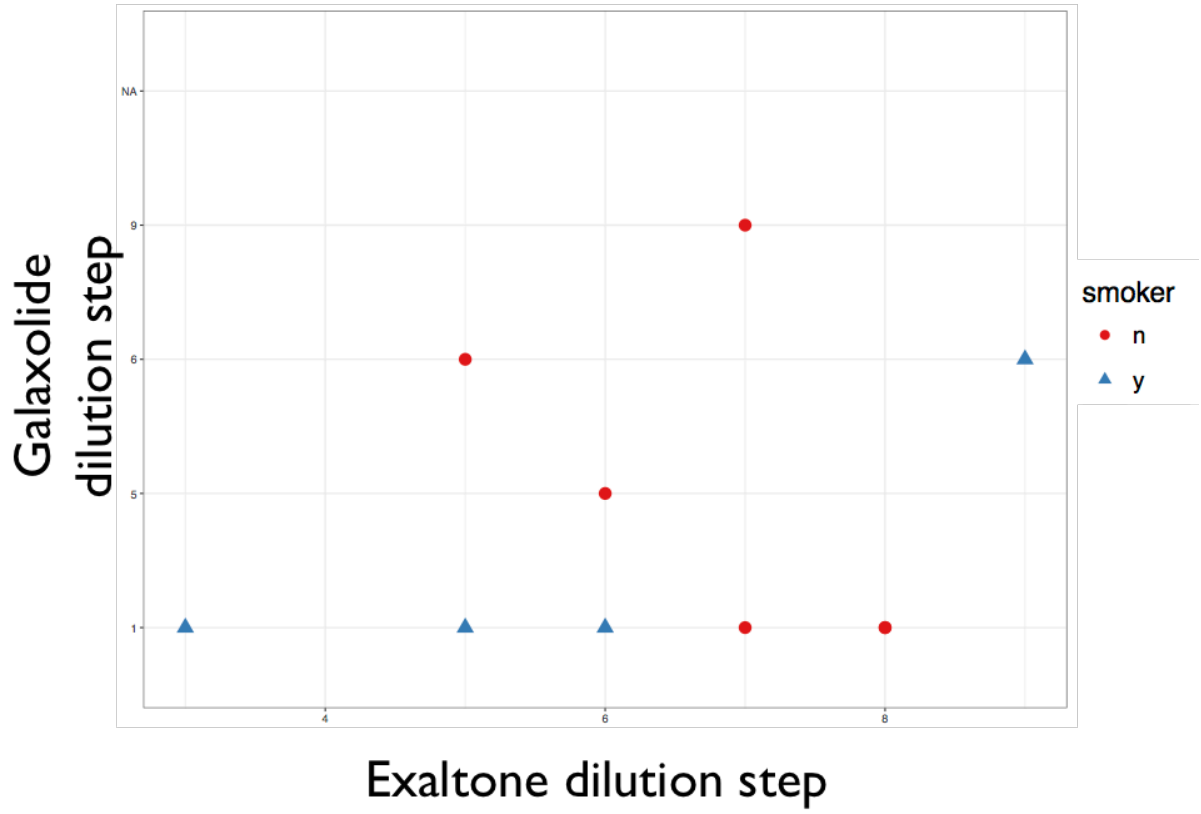



*Figure 96: Smokers and non-smokers are distributed throughout the sample.*

In Figure 96, the sample size is too small to calculate a reliable correlation between thresholds and smoking status, but it does not appear that the smoking status of a volunteer predicts their threshold.

**Musk Ketone and Habanolide Anosmia**

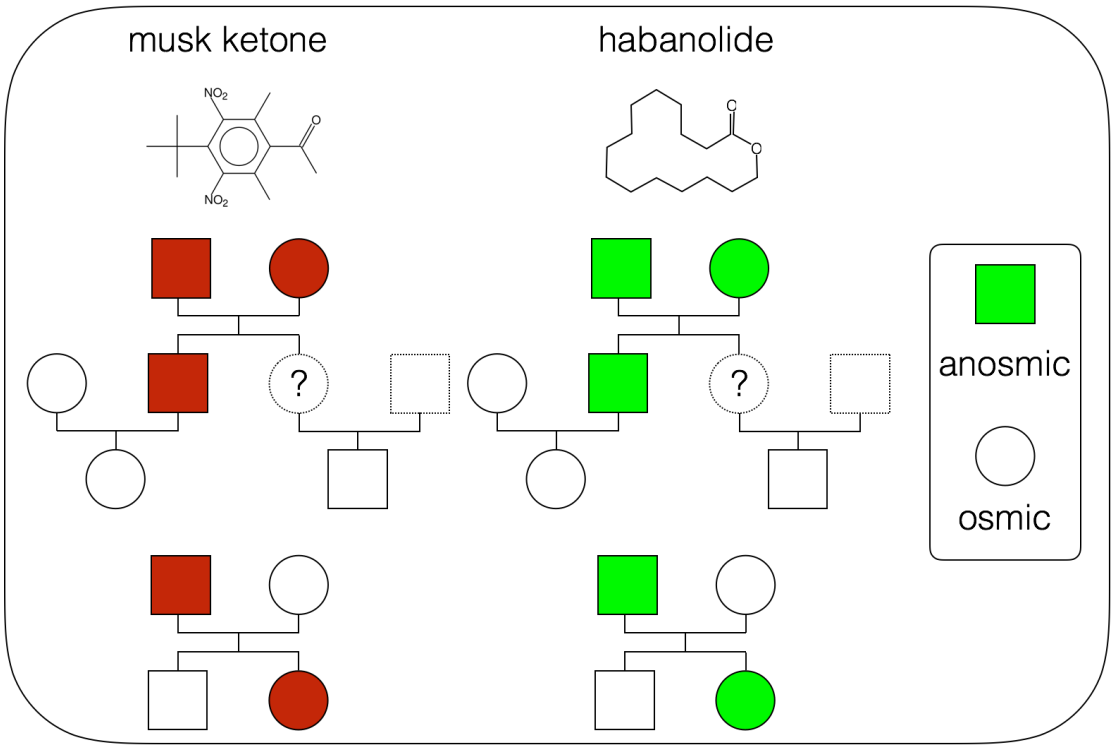



*Figure 97: Musk ketone and Habanolide anosmia overlap*

Musk ketone (a nitro musk) and Habanolide (a macrocycle) anosmia covary absolutely although the sample size is very small in this study.

**No Specific Anosmia for Exaltone®**

Although the thresholds varied between 10% and 0.1% for Exaltone®, there was no absolute anomia as was so clearly demonstrated for Galaxolide. The only outlier was volunteer 01/03, who could only perceive Exaltone at a high 10% concentration although this may represent a specific anosmia as defined by Amoore, i.e. a threshold raised above the normal by at least three standard deviations.

**Triangle Testing at Maximum Concentration is a Sensitive Predictor of Galaxolide Anosmia on Threshold Testing.**

If a subject can perceive Galaxolide at the maximum concentration (dilution step 1 - 50%) they will have a threshold many times lower than that, at least 2.5%, if not less. In any autosomal recessive inheritance pattern, some of these low threshold subjects are likely to

have at least one copy of the non-functional gene. In a dominant pattern they will have two functional genes. In either case the absolute anosmia is easily detected without using the complicated and labour-intensive threshold tests. Therefore, it is feasible to use just a robust test (multiple 2- or 3-AFC, R-index) at a high concentration (50%) to detect further anosmics, which we then went on to do for the extended family of family 3.

**Summary**

Although there does not seem to be an STV for Exaltone within the families, there is evidence for an STV to Galaxolide, Habanolide and Musk Ketone. This seems to be the result of an autosomal dominant or pseudo-dominant inheritance pattern. Further analysis of the genomes of the family members allowed me to correlate the phenotype of Galaxolide STV with variations in the olfactory receptor repertoire to establish a likely candidate gene as described in the following work.

## Single Gene Analysis

**OR5AN1 SNP Does Not Associate with Galaxolide Anosmia**

Several family members demonstrated a SNP (single nucleotide polymorphism), found at site 1014 (11:59,132,897 on the GRCh37 human genome map, or 11:59,365,323 GRCh38) with the exchange of a single cytosine for the expected guanine. This is a missense variant, resulting in the replacement of a leucine with phenylalanine at amino acid position 289. The shorthand for this is L289F, and is a previously described variant (HORDE database). The location is towards the cytosolic tail of the receptor and is unlikely to be involved in the binding site.

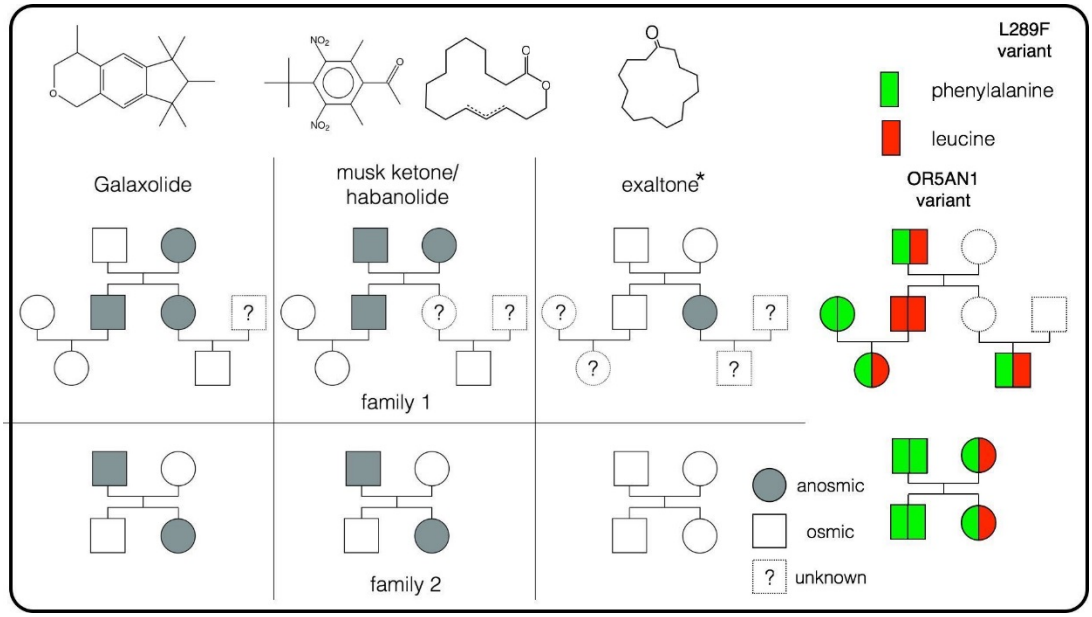



*Figure 98: Family phenotype and genotype for OR5AN1 L289F SNP*

However these SNPs did not segregate with the Galaxolide anosmia phenotype, consistent with the finding in the literature that this receptor is not activated by Galaxolide (Shirasu et al. 2014).

## Olfactory Exome Analysis

The receptor exome analysis provided a range of genomic variants in the Galaxolide anosmic group for family 1, shown in Figure 100. The analysis is on-going for families two and three. Within family 1, subject 01/05 (osmic and unrelated to all except 01/06) and 01/07 were not sequenced in time for this analysis.

### Galaxolide Anosmic Variants

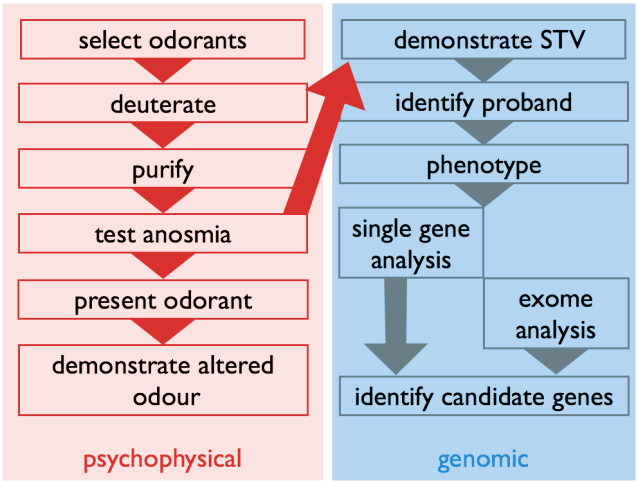



*Figure 99: Final step of the genomi strand of the thesis: candidate genes for Galaxolide anosmia*

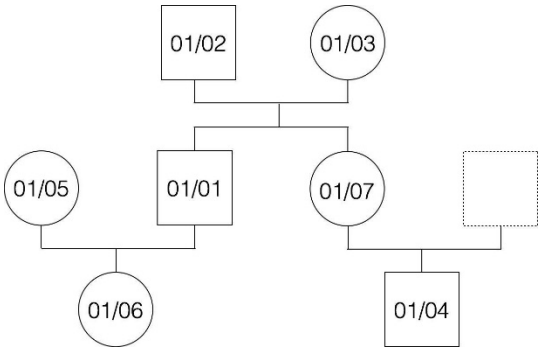



*Figure 100: Genogram and identity codes for family 1*

The genomic DNA variants enriched in the anosmic group were all olfactory receptor genes as shown in Table 24.

| OR gene | sequencing depth | effect | variant | Probable impact | other mutations | 1.01 | 1.03 |
|---|---|---|---|---|---|---|---|
| OR9K2 | 73 | Frameshift | NONE | HIGH | multiple | het | het |
| OR6C74 | 139 | stop codon | NONSENSE | HIGH | multiple | het | her |
| OR8U1 | 42 | Non-synonymous | MISSENSE | MODERATE | | het | HOM |
| OR4Q3 | 101 | Non-synonymous | MISSENSE | MODERATE | | het | het |
| OR4N2 | 108 | Non-synonymous | MISSENSE | MODERATE | | het | het |
| OR4K1 | 166 | Non-synonymous | MISSENSE | MODERATE | multiple | het | het |
| OR4E2 | 45 | Non-synonymous | MISSENSE | MODERATE | | het | het |
| OR6B3 | 32 | Non-synonymous | MISSENSE | MODERATE | | HOM | HOM |
| PLXNA4 | 144 | Non-synonymous | MISSENSE | MODERATE | | het | het |
| OR2A12 | 77 | Non-synonymous | MISSENSE | MODERATE | | het | het |
| OR2A2 | 83 | Non-synonymous | MISSENSE | MODERATE | two | het | het |

*Table 24: Gene variants in the OR exome present in the anosmic members of family 1 (01/01 and 01/03) and not in those family members able to smell Galaxolide.*

Table 24 is a list of the genes within the restricted search space of the OR exome which are present in one or both alleles of the anosmic subjects and not in the osmics. There are several obvious pseudogenes in the list. OR6B3 is the only homozygous gene in both anosmic volunteers.

**Galaxolide Osmic Variants**

The gene variants in the family members who can smell Galaxolide are summarised in Table 25.

| gene | Sequence depth | SNP | other SNPs | 1.01 | 1.02 | 1.03 | 1.04 | 1.06 |
|---|---|---|---|---|---|---|---|---|
| OR6N1 | 60 | F245L | | 0/0 | 0/1 | 0/0 | 0/1 | 0/1 |
| OR51G1 | 56 | Y125S | | 0/0 | 0/1 | 0/0 | 0/1 | 0/1 |
| OR5AN1 | 73 | L289F | | 0/0 | 0/1 | 0/0 | 0/1 | 0/1 |
| OR5A1 | 101 | D183N | | 0/0 | 0/1 | 0/0 | 0/1 | 0/1 |
| OR6S1 | 56 | R237H | | 0/0 | 0/1 | 0/0 | 0/1 | 0/1 |
| OR12D2 | 74 | V47F | multiple | 0/0 | 0/1 | 0/0 | 0/1 | 0/1 |
| RTP2 | 32 | G100S | | 0/0 | 0/1 | 0/0 | 0/1 | 0/1 |
| PLXNA4 | 144 | F40L | | 0/1 | 0/0 | 0/1 | 0/0 | 0/0 |

*Table 25: Variants in family 1 by ability to smell Galaxolide (anosmic subjects in green, those genes which are especially of interest are highlighted in orange.)*

There are no frameshift or stop codons, all of the variants are missense mutations and all are heterozygous, and the sequence depth is good for most. OR12D2 is almost certainly a pseudogene with multiple SNPs; the others have only a single amino acid variation.

Although they are all heterozygous and therefore less likely to be phenotypically relevant, there are two genes that are of interest (highlighted in Table 25): RTP2 and OR5AN1.

The sequencing of OR5AN1 is of greater depth and the distribution confirms the findings of my single gene sequencing by the Sanger process. Thus, the conclusions of that section, namely that it is not responsible for the Galaxolide STV, remain valid.

# Chapter Five: Discussion

## Chapter Synopsis

In the chapter that follows, I discuss the key experimental findings from the previous chapters: the psychophysical experiments with toluene, acetophenone and Exaltone; as well as the phenotyping and genotyping of the musk anosmic families. I discuss their strengths and weaknesses as well as the caveats to their interpretation in the support or otherwise of the vibrational hypothesis. I finally relate the findings to the current literature in the field and the potential avenues for further research.

## Toluene Psychophysics Pilot Experiment

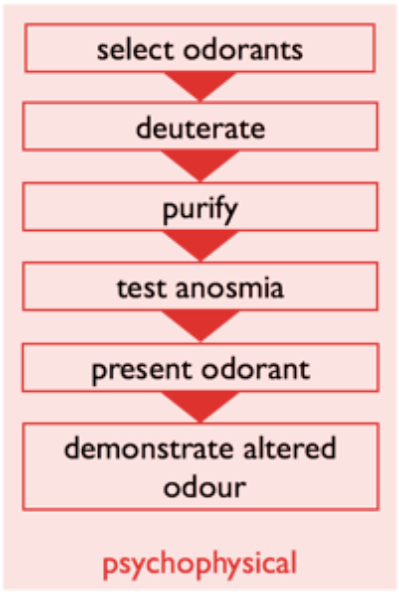



*Figure 101: Outline of psychophysical experimental plan*

Although the experiment followed the outline as in Figure 102, I could not demonstrate that unpurified toluene has an appreciably different odour character to its deuterated counterpart. The relative unpleasantness of the odorant meant that it was a poor choice for continuing the psychophysical experiments.

**Toluene Psychophysics: Strengths**

The method, however, seemed practical, robust, and relatively easy to replicate.

I identified several issues which need to be addressed by any further experiments: the need to preserve experimenter blinding, the bias introduced by sample selection without replacement, the ease of use of a simpler experimental design, and the need for multiple trials to achieve adequate power in a binary choice experimental design. This last requirement, together with the discomfort reported by the volunteers, meant a more pleasant odorant would be required. Using this as a pilot of the test method was successful.

**Toluene Psychophysics: Weaknesses**

The low number of trials and subjects meant that no inference could be drawn from the results, although they did not appear promising.

## Acetophenone Psychophysics: Key Findings

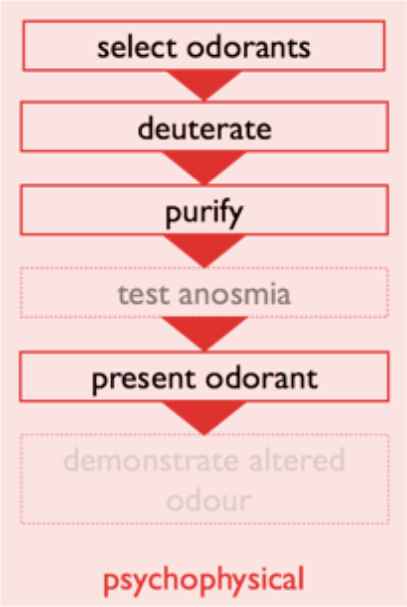



*Figure 102: Failure to show an alteration in the odour of isotopes for acetophenone*

The predicted, and previously reported (Turin 1996) altered odour in deuterated acetophenone using expert smellers, was not demonstrated as a shown in Figure 103.

Although there are some caveats and the tests were only conducted on a small sample, many trials (up to 400) were performed with each volunteer and no volunteer was significantly able to distinguish deuterated from undeuterated acetophenone at a rate greater than chance. We did not demonstrate the ability to distinguish odorants based on their deuteration state with acetophenone. This is consistent with previous findings (Keller & Vosshall 2004) but conflicts with Turin's own report and does not support the vibrational hypothesis.

**Acetophenone Psychophysics: Strengths**

The strengths of this experiment are the robust experimental method, with ultrapure odorants, randomisation and blinding. This excluded many of the previous sources of possible error from the previous work on the topic. (Keller & Vosshall 2004; Haffenden et al. 2001)

**Acetophenone Psychophysics: Weaknesses**

Turin (Turin 1996) mentions the different odour character of deuterated and non-deuterated acetophenone as being one of the supporting facts for his theory, without demonstrating that this was indeed the case. It may be that to certain noses there is an obvious change in character when the molecule is perdeuterated, but these experiments did not show that.

It may be that Turin was merely mistaken; after all he did not claim to have performed his analysis double-blinded and as Feynman said: 'the easiest person to fool...is you[rself]' (Feynman & Sackett 1985), but it was a mistake shared by all the perfumers tested in the sample. They all reported a more 'fruity' (or 'lower pitch', or similar) character to the deuterated sample when first presented with the two unblinded, yet were unable to reliably detect it in blinded tests.

Why then should this be? Is it possible that there is a perceptible difference but that all the volunteers failed to demonstrate that in this experiment?

An olfactory detection task is the summation of the noisy, chaotic, multiple inputs of the olfactory receptors to olfactory neurons, to the bulb, influenced by the higher centres as discussed previously as shown in Figure 104, to one output: 'yes or no'. Each of these steps should be considered in analysing why that task should fail.

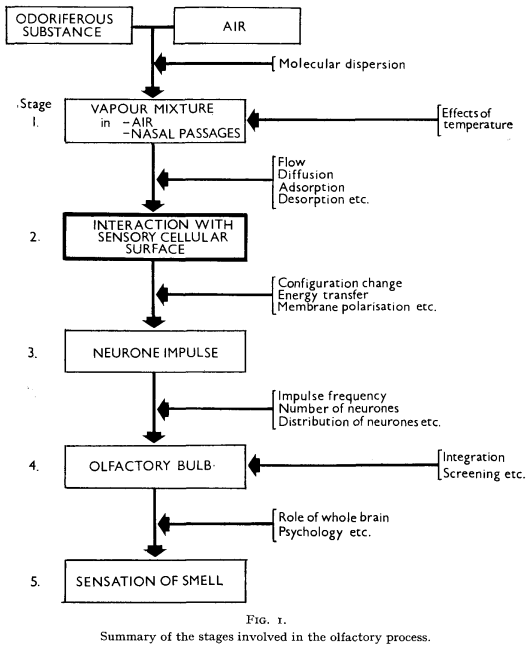



FIG. 1.
Summary of the stages involved in the olfactory process.

*Figure 103: The layers of the olfactory system, from Douek 1967 (Douek 1967)*

Pre-receptor level Causes

One cannot be certain that the deuterated acetophenone remains deuterated for long enough to reach the receptor perdeuterated. Hydrogen exchange and loss of the deuterium to environmental water begins as soon as the molecule leaves the GC. Diffusing into a very wet environment such as a mucus layer must only increase this loss. This does not explain why there is a perceived difference in odour for the initially presented samples.

Receptor Level Causes

At the receptor level, there are a few possibilities: the signal was lost in the receptor noise, there is a specific anosmia for the deuterated frequency in all subjects by chance, habituation of that receptor meant that the signal was lost after repeated exposures, or that there was competitive inhibition of the OR which 'blocked' the signal.

### A Relatively Weak Signal

Given that many receptors may be activated by one odorant, (Nara et al. 2011) it may be that there is a detectable receptor level difference in the activation step of certain receptors by acetophenone, but not others. One 'deuterated' receptor may well fail to be activated by the hydrogenated version, for instance, but among the multiple other receptors, all of which are activated to the same extent, the signal might be lost. The noise created by simultaneous activation of a number of receptors and their corresponding neurons at the bulb might drown the signal of the one or two neurons that detect a difference between the samples.

The signal could also be weaker because deuteration affects the vibrational characteristics of the CH bond, of which there are only eight among another eight C-C (plus the ring structure double bonds) and a C=O. It may also be that the DH bond vibrational frequencies are lost in the suite of other vibrations.

### Random Anosmia for Frequency

Another possibility is that, unlike in drosophila, (Franco, Turin, Mershin & Skoulakis 2011b) the deuterated-acetophenone detecting receptor is often pseudogenised in humans and that the small sample size by chance contained no-one with a functional version of this receptor. Given the 'unique nose' hypothesis of Menashe et al, (Menashe et al. 2003) it is not inconceivable that all the subjects might have a specific anosmia to

deuterated acetophenone, or rather a specific anosmia to the frequency of the bond within the molecule which is altered by deuteration. Until we have a better understanding of the olfactory genome and the structure-activity relations of the olfactory receptor, identifying this putative receptor is unlikely.

Although this possibility must be considered, as all subjects were similar, mostly male, ethnically Caucasian, and lived in south-eastern England, even with these factors, the likelihood of all six displaying the same, unrecognised, STV to only deuterated acetophenone seems very unlikely.

### Habituation

The reportedly obvious yet invisible-over-multiple-trials character difference may be explained by the quick habituation of the nose to acetophenone. Habituation, where the system becomes inured to the signal from a ubiquitous stimulus, may occur at multiple levels in the OSN, (Leinders-Zufall et al. 1999; Munger et al. 2001) bulb, (Wachowiak & Cohen 1999)and primary olfactory cortex (Poellinger et al. 2001). Acetophenone is said to habituate very quickly and it may be that the different signals are obvious on first sniff, but the distinguishing odour character/ receptor activation may not be present in following exposures. I can find no data on the rate of habituation for acetophenone, but as a crude analysis, the success rate of the first trial after a training set or having undergone previous trials showed that the subjects were no more likely to identify the relationship between the pair.

Volunteer MS's trials over multiple days are also a tantalising hint that there may be a habituation or inhibition effect. Although these constitute only a small amount of data which are not significant, the early successes may indicate that allowing an hour or more

between each sample improve the ability to detect the difference. There are concerns about sample degradation and blinding, making this technique impractical for use in most settings.

Habituation is a less likely cause of the finding as the odour of the samples was not reported as weakened throughout the experiment. No volunteer reported that they could not smell the samples at the end of a series of trials; they were merely unable to detect a difference in the odour of the two samples.

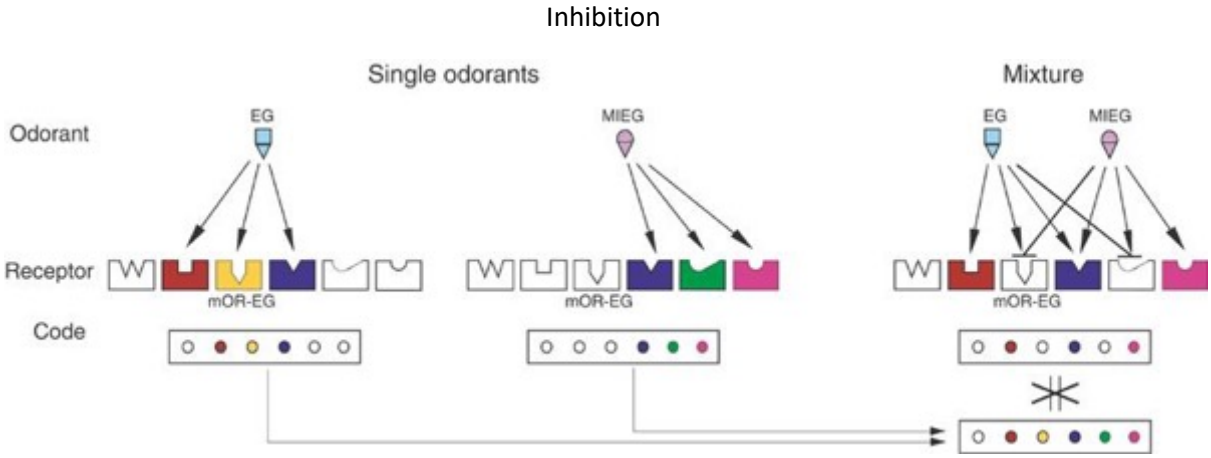



*Figure 104: Figure 5 from Oka et al (Oka et al. 2004). Demonstrating the complexity of a recognition task with a two-odorant mixture with competitive inhibition and only six receptors.*

Short-lived competitive inhibition of the olfactory receptor which detects the deuteration frequency by the hydrogenated odorant may be a possibility. After all, the odorants are exactly the same shape and should have the same affinity for the receptor binding pocket, but not the same ability to tunnel electrons by vibration.

Inhibition of a key receptor is suggested by the ability of most of the volunteers to successfully identify the relationship between the odorants when presented in a deuterated-first comparison (DvH) as compared to their ability with the 'same' odorant pairs. This is complicated by the bias to finding the samples 'the same' (i.e. the decreased 'yes-rate'), although the Tukey 'honestly significant variance' shows a significant difference between the success rate in DvH compared to HvH and DvD. Individual analysis showed that this

difference became greater over multiple sessions for two out of the three volunteers with multiple sessions (TG and SG).

The question arises as to whether the odorants from the previous trial might have influenced the results of any given pair comparison: that is, whether the second part of DvH or HvH comparisons influenced the success rate of a subsequent trial. Performing such an analysis is relatively trivial but the variation in the time between trials makes the results unreliable. For most trials, the time between trials was between 30s and one minute: identifying the odorants under the UV light, recording the results, and replacing the sample. Between others it would have been longer for various reasons, and I did not always record this. Any inhibition would cease with clearance of the odorant(s) and would probably act on the order of seconds. In the first published description of odorant antagonism (Oka et al. 2004) the neural activity of the odorant is approximately 30s for most measured neurons (the time scale in the results makes more accurate estimates difficult) but as the odorant mixtures were directly applied and not presented to the nose, it is difficult to know what the time scale is like in normal mucosa. For these reasons, I did not look for inhibition between pair trials.

The interesting predilection in most volunteers for reporting a difference in the odour of the samples must be considered in examining this finding. If a volunteer replied 'no' to every sample comparison, the DvH and HvD scores would be 100%, whilst they would be wrong for every single HvH or DvD comparison. With the vagaries of chance and the small sample size, this may be the explanation, although it is striking that this finding is only significant for the DvH comparison in multiple volunteers.

Post-Receptor Factors

Habituation occurs within the neuron and both inhibition and habituation occur within the olfactory bulb as part of the processing of the olfactory signal. Either of these locations could be a source of difficulty in the odour comparison task.

An indication of how complex a task is, comes from the observation of a marked bias in over half of the volunteers against reporting that they perceived the samples to be 'the same'. This 'yes-rate' was decreased by exposure to the training set but was not significantly affected by professional experience.

This bias must be the result of central factors. All the volunteers were aware of the underlying premise of the experiments and aware that, if the vibrational hypothesis were correct, they were expected to be able to tell the difference between some samples. Perhaps this lowered the 'set point' for comparison between the samples and made them more likely to interpret the stochastic variation in noisy olfactory input as a difference in the odour character. However, all the volunteers also knew that the samples were randomly presented and that any one sample pair could be the same or different with equal chance. Perhaps this knowledge did not factor as strongly into each individual judgement as the desire to 'tell the difference'.

The main finding of this experiment is striking: there is no perceptible difference in the odour of deuterated and hydrogenated acetophenone, which was not demonstrated by subjects' belief that they could smell a difference. The volunteers believed they could detect a difference in the smell of the two odorants in a pair, even if that difference was, according to the results of the experiment, not present. So what was that difference? If there were a monotonic, linear path from detector to output with a clear 'yes' or 'no' answer for every comparison, there could be no possibility that central factors would be able to shade the

result. Subjectively, the smell of even a single molecule is exceptionally complex, and obviously variable, even in the most strictly controlled conditions. This demonstrates what a complex task odour comparison is, and that the apparently simple question of whether these samples smell the same requires a hugely complex process to answer.

**Auto-Experimentation**

In the acetophenone experiments I was the sixth experimental subject, SG. Self-experimentation has a long history, especially in the medical sciences, resulting in significant discoveries and at least twelve Nobel Prizes (Weisse 2012), the last of which was awarded to Barry Marshall in 2005 (nobelprizeorg n.d.) (the year after Axel and Buck were awarded theirs) for his demonstration of the infectious cause of stomach ulceration: Helicobacter pylori (Marshall et al. 1985). A review article in 2012 (Weisse 2012) demonstrated no clear guidelines or instruction from regulatory agencies and suggests that autoexperimentation, if anything should be saluted for demonstrating experimenter commitment to the project and willingness to undergo anything that their subjects do. Neuringer (Neuringer1981 n.d.) identified five potential problems with self-experimentation, but only one applies to this experiment: "experimenter expectations", in which knowledge of the experimental hypothesis biases the results, which I addressed with the thorough blinding procedure.

**Acetophenone Psychophysics: Conclusion**

Although acetophenone is a pleasant, easily available odorant, it is small and is therefore probably detected by numerous different receptor types. I decided to examine another odorant using similar methods.

## Musk Psychophysics: Key Finding

This experiment confirms a prediction of the vibrational hypothesis: deuteration of some musks alters their odour character to humans. Trained odour evaluators detect a common ‘deuterated’ odour in all four of the musks which were catalytically deuterated. A

mixed group of mostly naive smellers were objectively and significantly able to smell a difference between such a musk and its sham-deuterated (hydrogenated) counterpart in a double-blind trial.

**Musk Psychophysics: Strengths and Limitations**

The strengths of this experiment are the use of ultrapure preparations of samples, a double-blind technique and randomisation of presentation.

As with acetophenone, there are limitations: possible sources of error which were not or could not be controlled in this experiment. These could have arisen from molecular and non-molecular features.

Sources of error: Odour Alteration Other Than Molecular Features

Although there was an altered odour, there are several ways in which this odour could have been the result of something other than the molecule being examined, such as sample contamination or blinding failure.

Sample Contamination

Human noses are better than is commonly supposed (Shepherd 2004) and can detect very low concentrations of odorant molecules. Even a very small amount of the right contaminant may alter the odour of a sample. Commercially available sources of rarer molecules, such as deuterated forms, are often only of 95% purity due to economic considerations.

To combat this all samples were purified using a perfumery-standard preparative gas chromatography.

The use of odorants which have gone through the same catalytic process (deuteration and sham-deuteration) should increase the likelihood that there are no contaminants which are present in one set of samples but not the other. In addition, the likelihood of any of

these contaminants having the same transit time through the GC as the odorant in question is extremely small. However, it is impossible to entirely exclude a contaminant with a coincidental similar transit time to one or the other of a probe molecule pair. Gas chromatography purification has been said to be as close to 100% pure as it is possible to get in the real world and any contamination of the samples after decanting into the Eppendorf tubes was strictly controlled for.

### Blinding Failure

The double-blind procedure was, if anything, slightly excessive. Removing the experimenter from the sample-smelling procedure completely should remove any believable interference. Even with the experimenter present, the samples were still impossible to tell apart. However, as I arranged the tubes randomly in the heating block, I could have potentially followed the movements of each tube through the randomisation process and known which was which. Even unconsciously this should have influenced the outcome, although as the subjects could not even see me, such influence is very unlikely.

### Sources of error: Odour Change is Due to Non-vibrational Molecular Features

The experiment demonstrates conclusively that the samples I prepared possessed an altered odour character to most volunteers. Although there is an altered character and this character might arise from the deuteration of the molecule, the odour may be altered by mechanisms other than the vibrational hypothesis.

The main concern is that deuteration alters the chemistry of a molecule and there is no doubt that there is significant chemistry in the olfactory mucus and epithelium.

### Odorant Binding Proteins and Transport

The precise steps of molecular transport through the olfactory mucus are still unknown, as is the role of the olfactory binding protein. It is possible that deuteration alters

binding affinity and transport of the molecules by the OBPs and this is what alters the smell character.

### Enzymatic Changes

Within the olfactory mucus there are multiple enzymes. The raspberry odour of *4,8-Dimethyl-5-(1-methyleneethenyl)bicyclo[3.3.1]non-7-en-2-one* and its variation in the presence of the enzyme CYP2A13, as discussed in the literature review, demonstrates the importance of these in odour character. The characteristic smell of a musk could be the summed odours of a specific set of breakdown products within the mucus. A difference in catalysis due to substrate deuteration with different breakdown products could therefore be the source of the odour change.

### Hydrogenation of Deuterated Samples

Finally, we cannot even be certain that the musk reaches the receptor with the deuterium intact. All deuterated molecules begin to exchange deuterium for hydrogen with water in the environment from the moment they are synthesised. This was a significant constraint on the sample shelf-life, once purified. This loss of deuterium should, however, result in a loss of altered character rather than the generation of a spurious difference. Musks are relatively large molecules and have more deuterium to lose than acetophenone, which may be another explanation for their odour character to be altered when acetophenone's is not. However, this loss of deuteration in a watery environment is a concern for further *in vitro* work.

**Why Musks But Not Acetophenone?**

Why did the musks show an alteration in character when deuterating acetophenone did not? These experiments controlled for some of the possible reasons, as discussed in the previous chapter: receptor noise, specific anosmia to the altered character, habituation, and small number of C-H bonds affected.

It has been shown that the musks likely stimulate only a few receptor types; probably around two in most people (Nara et al. 2011). Signal competition from other OSNs detecting the musks is less likely in this single or low-receptor environment, and any alteration in signal is more likely to alter the whole percept and allow this to be made available to the consciousness of the observer.

All the subjects were tested to confirm their ability to smell musks, and a larger cohort was recruited, making the inadvertent collection of an unknown specific anosmia unlikely.

Musks do not habituate as quickly as acetophenone so the risk of loss of altered character in multiple trials was not as great. Musks are also bigger: acetophenone has eight C-H bonds while Exaltone has 28, and therefore deuterating most or all of these may have a greater impact on the overall odour. The demonstration that the altered odour character was only present in molecules with a greater than 50% deuteration seems to support this.

**Summary of Psychophysics Results**

One of Hettinger's (Hettinger 2011) objections to the Franco (Franco, Turin, Mershin & Skoulakis 2011b) demonstration of vibrational olfaction in *Drosophila* was that humans do not smell in the same way as flies. It seems unlikely that such a highly conserved structure and evolutionarily important function should differ in such an important mechanism, but it must be granted that this is possible, and therefore the demonstration of a vibrational component in *Drosophila* olfaction by Franco et al (Franco, Turin, Mershin & Skoulakis 2011b) may not apply to human olfaction. These experiments have demonstrated very clearly that humans do detect an alteration in odour when the right molecules are deuterated.

It is not possible to completely prove that the alteration is due to a vibrational sense given the caveats previously discussed, but it does at least remove one more objection to the theory. Furthermore, if an alteration in the scent of a musk molecule occurs when deuterated, and the odorant is very likely to be detected by one or two receptors, the receptor for that molecule is the best target for further investigation, if only that receptor can be identified, the objective of the second part of this thesis.

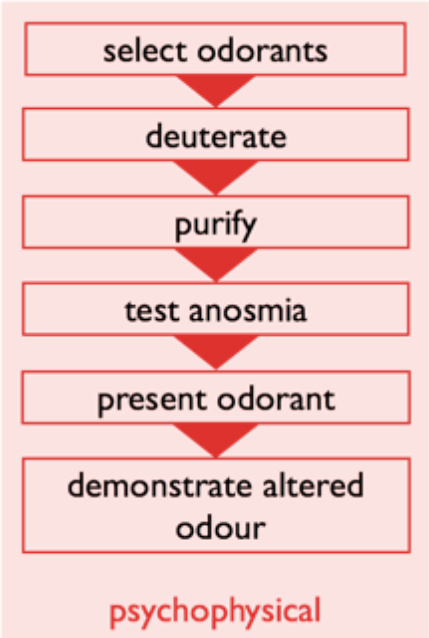



*Figure 105: Psychophysical demonstration of altered isotopic odour*

## Response in the Literature

To explain the results of the Exaltone experiment in Gane et al (Gane et al. 2013), Block et al (Block, Jang, Matsunami, Sekharan, et al. 2015) have concerns about the purity of the samples. They point to an impurity peak at δ 0.84–0.87 on the original NMR shown in Figure3.6 and discount the GC purification step, stating that '…it is not known whether the products responsible for the additional δ 0.84–0.87 impurity peak, or decomposition products of the compound(s) in the hot injection port, coelute with deuterated musks.'

Turin et al replied,

*"Block et al. describe the same impurity in their synthesis, and it is visible in their NMR spectra (Figure S2.6 of ref. 1) at 0.87. This impurity does not coelute with the musk and is shown and correctly labelled in Figure 2 of ref. 2. Furthermore, our sham deuteration protocol controlled for this. We therefore remain entirely confident that the difference in smell between isotopomers revealed by our double- blind trials is because of the pure peak of deuterated musk. "*

In their reply to this, Block et al claim that the peak in their spectra at1.26ppm was the residual protons in cyclopentadecane-d30 and reiterated that this was a potential confounder. This is obviously a consideration but it seems unlikely that all of the other demonstrations of vibrational olfaction, although admittedly in insects, also had a similar co-eluting contaminant.

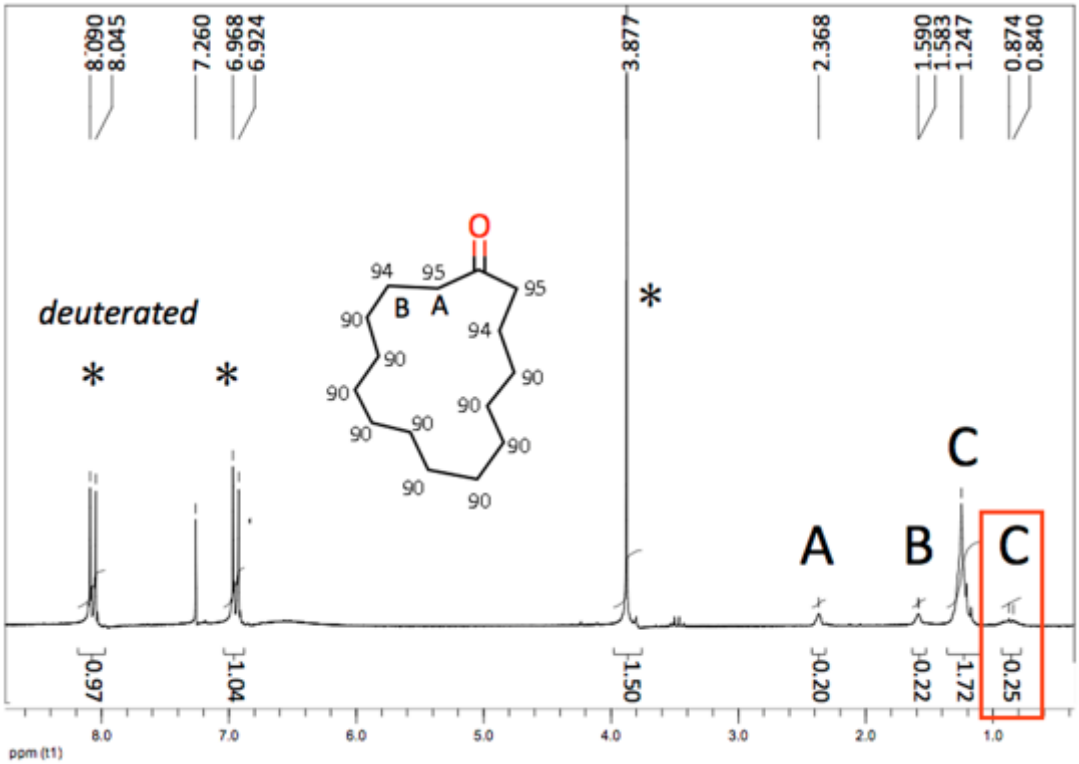



*Figure 106: Detail of Gane et al Figure 1, putative impurity peak highlighted from Block et al (Block, Jang, Matsunami, Sekharan, et al. 2015)*

**Problems With the Block et al Experiments**

The Block paper did the Turin/Dyson/Wright theory '…the service of taking it seriously', as Dr Andrew Horsfield has put it (pers. com.). The Turin modification of the 'undulatory' hypothesis is that the vibrations were measured in the receptor by an electron tunnelling process, which was the first set of experiments to check the predictions of the model at the receptor level. The paper makes reference to the published version of the experiments in the previous chapters (Gane et al. 2013).

As with all scientific endeavour, one must weigh the balance of probabilities with the main finding of the paper: that in the heterologous expression systems (a standard tool in human olfactory biology) it was not possible to demonstrate a difference in luciferase activity between deuterated and non-deuterated odorants is a powerful one against the vibrational hypothesis. That said, Turin et al (Turin et al. 2015) state in their reply: 'there are many ways of obtaining a negative result, not all of them interesting.'

However, there are some flaws in the experimental portion of the study, both in the first receptor complement screening; and in the second individual receptor experiments.

General Concerns with In-Vitro experiments

There is no doubt that receptor expression systems have advanced the field enormously. The question remains that given that the systems must be manipulated in sometimes fairly significant ways to allow the measurement of OR activity, does this manipulation alter the biological activity of the receptors? In other words, can we rely on the output of the expression systems as being a reliable representation of OR function?

Several projects have examined this variation and extrapolation from heterologous expression system data must take into account at least three differences between the biological olfactory system and the isolated receptor: the lack of the rest of the biological

system, (Spehr et al. 2003), (Sanz & Schlegel 2005), (Matarazzo et al. 2005), (Oka et al. 2006) alteration of the downstream signalling system, (Shirokova et al. 2005) and the possibility of omitted alternative activation pathways(Simmons 2005).

Oka et al (Oka et al. 2006) compared the different methods of ligand assays for the olfactory receptor MOR-EG and others: *in vitro* using HEK293T with both Golf and Gα15, *ex vivo* calcium imaging of dissociated labelled OSNs, and *in vivo* intrinsic imaging of the bulb (Oka et al. 2006). They found that there were significant variations in, for instance, the thresholds for vanillin. When heterologously expressed in HEK293 and when present in dissociated OSNs, the MOR-EG receptor displayed similar responses to vanillin and the receptor's most well-known ligand, eugenol; but when the pair's relative activation of the MOR-EG glomerulus was assessed by both intrinsic signal and calcium imaging, eugenol gave a strong activation, but vanillin barely registered.

If we accept that the three-dimensional pattern of activation of the OB, and that the 'odour map' is the representation of the percept of a smell in the brain, this brings caution to the interpretation of psychophysics as a method to probe the receptor mechanism: the receptor level and whole-animal level activity may not agree. However, this caveat only works one way: a signal may be *lost* but not *generated* by this discrepancy. If there is no difference in the smell of two molecules then it must be caused by something, although that something may be the generation of new molecules by a chemical process within the mucus.

<u>In-vitro Experiments: Functional Selectivity</u>

The removal of olfactory mucus allowed vanillin to be detected by the MOR-EG receptor in Oka's work, but vanillin is easily detected by mice and humans with an intact mucosal layer (Eccles et al. 1989). What then could be the reason for a decrease in sensitivity in the presence of mucus at this glomerulus? A simple partitioning model where

the local concentration is reduced fails to explain why the threshold is still so low for the whole organism. One possible explanation is perireceptor metabolism of vanillin into an odorant which is detected by some other receptor, not MOR-EG. It could be that it is this second receptor is the receptor that we humans associate with the percept of vanillin i.e. the 'smell of vanilla', not MOR-EG; but there is another factor: functional selectivity.

There is now extensive evidence that different agonists can bind to the same GCPR receptor and activate different intracellular pathways, perhaps through different conformational changes of the receptor (Ghanouni et al. 2001) or perhaps mediated by different G-proteins linked to the same receptor (Sivertsen et al. 2013). This has been shown to be pharmacologically and biologically relevant for a number of receptors, including the histamine (Alonso et al. 2015) and ghrelin (Sivertsen et al. 2013) receptors.

The importance of this work is that the affinity of the ligand for a receptor can no longer be considered to be a direct indicator of its activation of the intracellular signalling pathway and that the exact mechanisms of activation, even in pharmacological systems, are still not understood. This fact should bring caution to the interpretation of data from heterologous expression systems, as these systems may not have the alternative intracellular signalling pathways intact.

Block et al: Expressed Receptor Screening

For the receptome-wide screening the Matsunami process has only been able to express 330 of the estimated 420 functional human receptors. Approximately 23% of the known human ORs are not represented in the assay at all. This leaves the possibility that other receptors would have reacted to the deuterated or non-deuterated odorants, although they were not included in the assay.

The researchers do not state the concentration of any of the odorants they use during the screening process. The detailed concentration-response curves are only given once the receptors have been identified. The variable dose-responses between the expressed receptors in this study and many others (Keller et al. 2007; Nara et al. 2011) are well-known. At only one concentration the screen may have missed other muscone receptors which may be relatively insensitive to the odorants but which are still differentially sensitive to only the deuterated or non-deuterated ligand.

The graph of OR response to the screening odorants demonstrates some other higher-response receptors. These are identified in a response as OR5E1 and OR5M9, neither of which responded to the deuterated or non-deuterated Exaltone (Block, Jang, Matsunami, Batista, et al. 2015). The explanation for the false positive was that there was a 'high receptor background' for the receptors. If the receptor expression is so variable, it may be that there is a similar 'low receptor background' giving a false negative.

**Individual Receptor Experiments**

For the individual expressed receptor experiments, which represent the most powerful evidence that there is no variation with deuterium or 13C isoptomers, several caveats apply.

Although the Matsunami expression method is recognised as a gold standard of olfactory receptor expression and functional identification, it does force the expression and trafficking of the receptors through the use of the RTPs and other proteins.(Zhuang & Matsunami 2007) These proteins do not appear to affect the function of the receptors (Mainland & Matsunami 2012). Although these systems have been correlated with the functions of the receptor in humans, (Keller et al. 1998) as discussed in the specific anosmias section, they have not been shown to directly correlate with other means of

receptor activation - dissociated neurons for instance - although they have been correlated with functional imaging of the olfactory bulb (Oka et al. 2006).

This non-neuronal environment has some drawbacks. As Block et al state, it may be that there is no electron source in the expression system. As Turin said in his 1996 publication '...future attempts to express or reconstitute receptors should include...an electron donor in the cytoplasmic phase before testing for response to odorants.'(Turin 1996) Additionally, a possible alternative activation pathway for functional selectivity may not be detected in the forced-expression system with only a cAMP luciferase readout.

Although there has been evidence for some time that there may be complex temporal and spatial aspects of GPCR signalling (Lohse & Hofmann 2015), this system focuses on a simple linear steady-state signal which of course may not be the reality *in vivo*.

The recent demonstration of a copper-coordinating requirement for some ligand-receptor pairs (Duan et al. 2012) by many of the authors of this paper leaves the question of which other heavy metals or other requirements that we do not understand are excluded from this assay. These copper-coordinated ligands were tested in the presence of copper in Block et al and did not show a variant response to isoptomers. Turin originally suggested that zinc might have a role to play as an electron acceptor, although he has since downplayed the possibility [Turin: pers. comm]. The lack of olfactory mucus and OBPs is also noted by the authors as a possible source of variation.

The authors do not comment on another possibility; that the isotopes may act as inverse agonists. The OR has a low constitutive activity, as discussed in the previous chapter. One or more of the test agonists may act to reduce this activity in a receptor but this would not be detected by the assays used.

Citing their focus on the receptor-level experiments, the authors refuse to comment on the psychophysical aspects, including the smell of their compounds (Block, Jang, Matsunami, Batista, et al. 2015). This has two problems. The first is that their charge that impurities were present in the Exaltone experiments which led to the alteration in odour is not valid if their supposedly purer samples also smelled different. The second is that the d30-muscone is analysed as not possessing any vibrational frequencies within what Turin (Gane et al. 2013) tentatively identified as the 'musk bands' 1380-1550 $cm^{-1}$. If this no longer smelled 'musky' this would be very important, but they refused to comment (Block, Jang, Matsunami, Batista, et al. 2015).

In conclusion, these results were the investigation that the vibrational hypothesis deserved and the experimental plan similar to that of this project was performed with much greater resources. Unfortunately, the findings were entirely negative, and although there are concerns with some of the experimental portions (I am unable to comment on the theoretical aspects), the balance of probabilities must be considered to have shifted against the vibrational hypothesis. Block et al do call for further genetic studies researching more musk receptors in humans, which this project has done.

In the reply to the paper, (Turin et al. 2015) we pose the question of what mechanism the behavioural studies demonstrate.

If the vibrational hypothesis is not in fact a good working description of the ligand-receptor activity for ORs, it is worth considering the alternatives. The isotopic alteration of association/dissociation curves and on/off rates has been shown not to be an issue by this study, and impurities are unlikely to co-elute for all of the isoptomers studied.

The only realistic alternative explanation is peri-receptor events. Isoptomers display altered binding to and therefore metabolism by olfactory mucus enzymes or OBPs.

Metabolism may form one or more by-products which also act as agonists, partial agonists, or antagonists to the same or other receptors. Altered trafficking or binding by OBPs may alter the activation and sensitivity of the receptor for the isoptomers in a way which alters the perception of their odour.

The Block et al paper only confirmed that the OR5AN1 receptor reacted to musks and no other human OR was shown to do so. However, as discussed in the literature review, there is abundant evidence to suspect that there is at least one more receptor in the human receptome. This may be the one to show altered activity to deuteration. The sequencing of Galaxolide-anosmic families would point to a possible candidate for this.

## The Genetics of Musk Anosmia in Families

The vibrational hypothesis is a description of the physics of the OR-olfactant interaction at the atomic level and the mechanism of GPCR activation.

Behavioural tests are an important preliminary step in assessing the credibility of the theory, and a valuable way to identify specific case studies to investigate more rigorously. The previous work demonstrated the alteration of the odour character of Exaltone in humans; the next step is to confirm that this is a receptor-level phenomenon.

Identifying the receptor that is primarily responsible for the detection of the musk Exaltone would allow the closer interrogation of its function, and therefore more evidence for, or against, the vibrational hypothesis.

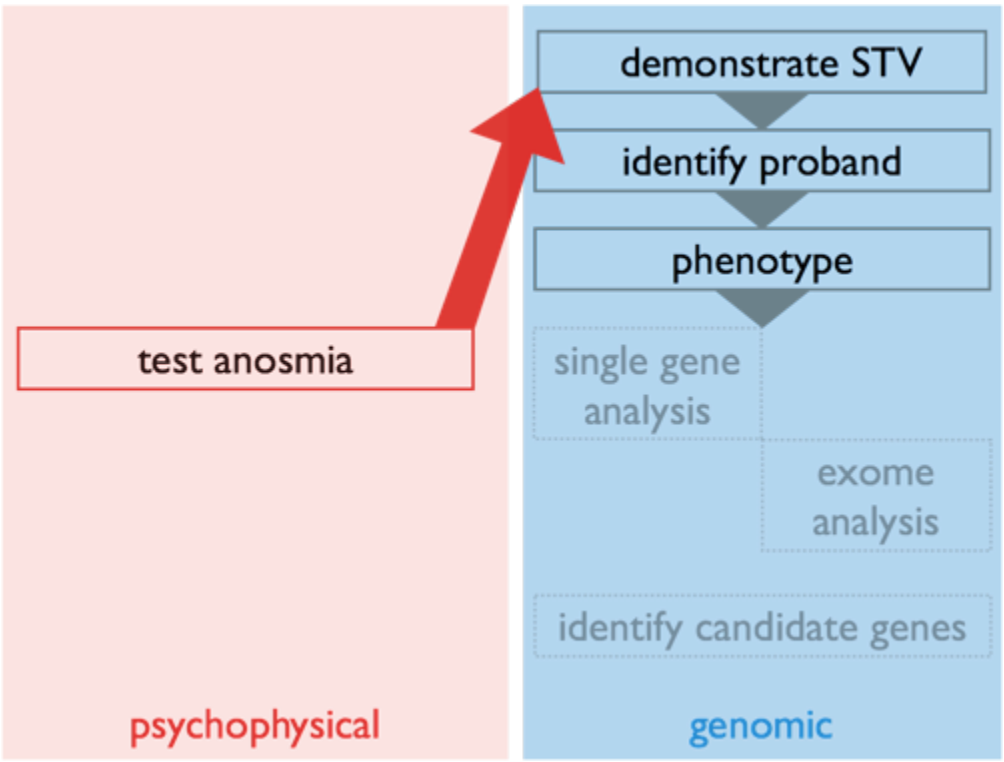



*Figure 107: Successful demonstration of a specific anosmia lineage*

**Phenotype: Inheritance Pattern**

Several conjectures can be supported merely from examining the distribution of the phenotype within the families and correlating the threshold tests as graphically demonstrated in Figure 109.

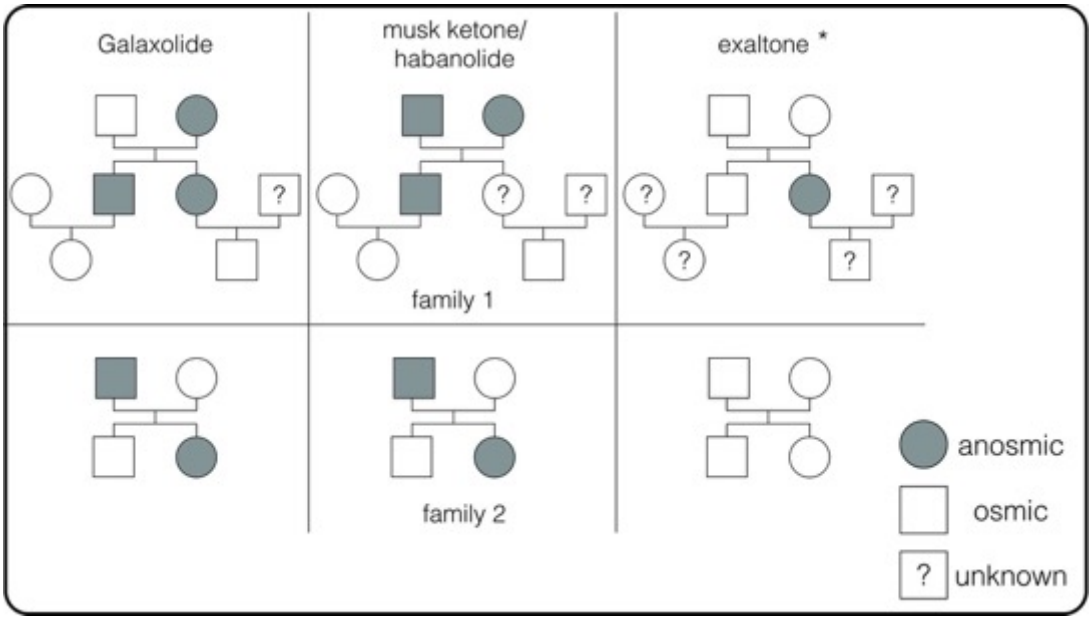



*Figure 108: Perception threshold of Galaxolide does not coincide with that of Exaltone*

The relatively high population prevalence and transmission within the families make a high penetrance genomic trait extremely likely. Assuming such a Mendelian trait, the small number and small size of the families within study make the inheritance pattern difficult to discern with a high degree of confidence. Since there are slightly more female than male anosmics, an X-linked condition is the most unlikely. If the anosmia is the result of a single receptor pseudogene, an autosomal recessive pattern would be the most likely. The genograms do not rule this out but the inheritance of the anosmia by both of the second-generation children in family one is unlikely (Mendelian inheritance chance of 12.5% if the father in the first generation is not a carrier). An autosomal dominant inheritance is not possible in a receptor pseudogene, as there are two alleles for each receptor. At most the loss of a functional single receptor gene might be expected to lead to an under-expression of that receptor and a raising of the threshold for that specific ligand, but as there is a complete loss of function (total specific anosmia for the target olfactant), this too seems unlikely. Although the exact mechanism behind olfactory receptor expression patterns within the OSN and mucosa is not known, non-functional receptors are not expressed (Markenscoff-Papadimitriou et al. 2014).

There is a preservation of general olfactory ability as measured by UPSIT or Sniffin' Sticks™ across the volunteers, and therefore a single point of failure for a single (or small group) of odorant(s) is most likely to be at the receptor or pre-receptor level. Given this, the most likely pattern of inheritance is a pseudo-dominant pattern, where the incidence of a defective allele is so high in the population that there are many carriers. In the pseudo-dominant case, the condition still requires the inheritance of two defective alleles of the gene: an autosomal recessive pattern. However, because of the high chance that one of the parents is a carrier, the Mendelian pattern on the genogram appears to be dominant when assessed across multiple families. This pattern is what would be expected in an olfactory

receptor pseudogene with low evolutionary conservation pressure. The very fact that three unrelated, although compatriot, families were relatively easily identified suggests that the gene is common: either the functional gene is not under evolutionary pressure and can therefore spread through the population, or the loss-of-function is strongly conserved for some reason. Several perfumery trainers have independently suggested to me that the rate of musk anosmia in the general population is approximately between 1-5% (J. Stephen, R Duprey, pers comm), although a recent analysis of OR expression within the WHOM (whole human olfactory mucosa) in 26 individuals showed that all expressed the muscone receptor OR5AN1 (Verbeurgt et al. 2014).

**Thresholds for Galaxolide and Exaltone® Are Not Correlated**

Although the first subject identified reported an Exaltone anosmia, threshold testing did not show this. In fact, he showed a threshold of 0.25% on testing. He was however completely anosmic to all other musks tested. The same mismatch was true for most of the volunteers. The thresholds and triangle testing showed a very poor correlation between the perception of Exaltone and the other musks. This suggests, consistent with the literature, that there are multiple musk receptors in humans.

**Musk ketone and Habanolide Anosmia**

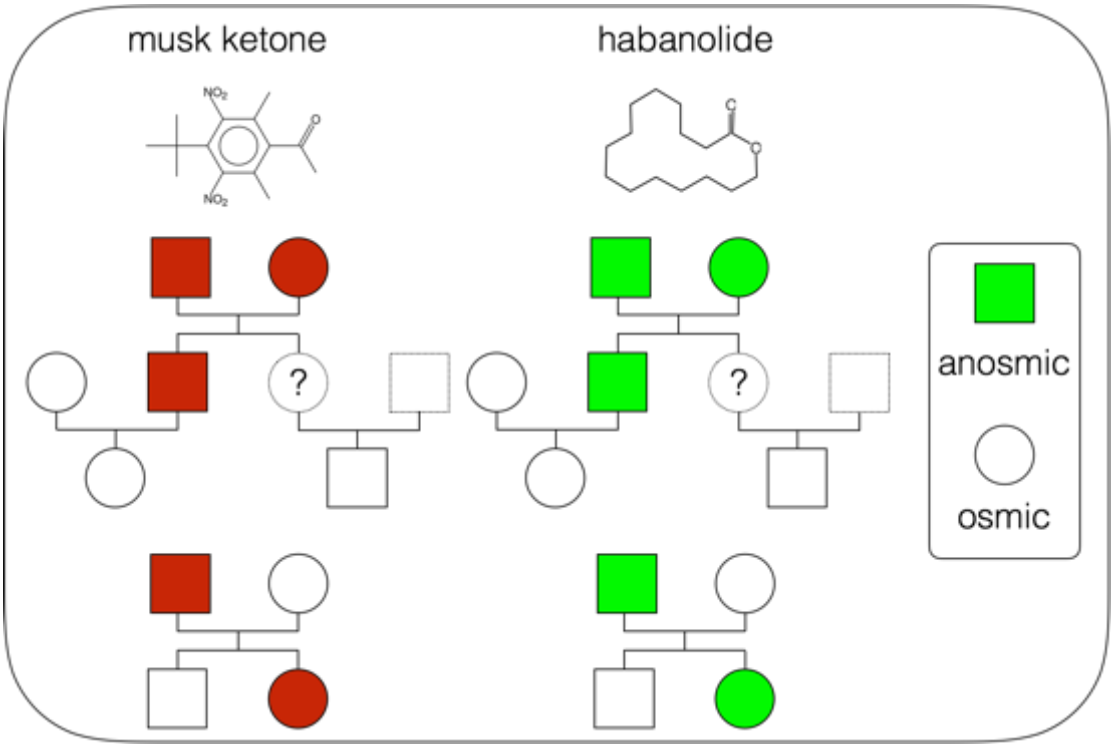



*Figure 109: Musk ketone and habanolide anosmias coincide*

Musk ketone (a nitro musk) and habanolide (a macrocycle) anosmia covary absolutely although the sample size is very small in this study. This particular association has not been reported in the literature but Gilbert (Gilbert & Kemp 1996) showed an association of an hyperosmia to a nitro, musk xylol, and Exaltone, a macrocyclic musk, which was not demonstrated here.

The Galaxolide anosmia overlapped with this anosmia but one subject (01/02) could robustly detect both habanolide, musk ketone, and Exaltone, but not the Galaxolide. Similarly, one subject (01/02) could detect Galaxolide, but not habanolide or musk ketone.

Although the sample sizes are small, I find it likely that the anosmia to both musk ketone and habanolide are a result of the same defect in the olfactory system, be it receptor or otherwise, but distinct to that of the Galaxolide anosmia, which would be a fruitful area for further research.

**No Specific Anosmia for Exaltone®**

Although the thresholds varied between 10% and 0.1% for Exaltone®, there was no absolute anosmia as was so clearly demonstrated for Galaxolide. The only outlier was volunteer 01/03, who could only perceive Exaltone at a high 10% concentration although this may be classified as a specific anosmia as defined by Amoore (Amoore et al. 1968). Amoore's classification of a specific anosmia required a three-standard-deviation difference in threshold, which is not possible to calculate reliably on a small sample such as this.

Once again there seems to be more than one musk receptor, as a complete STV for one musk does not predict the STV for another.

This lack of an Exaltone STV was a little disappointing, as identifying the receptor responsible for the altered odour of Exaltone when deuterated was the aim of this experiment. However, finding at least one of the musk receptors would allow for expression testing with that receptor. Galaxolide was, unfortunately, not one of the musks originally trialled as deuteration targets but would be possible to deuterate using the methods described, even though it contains double bonds, as the double bonds are contained within an aromatic ring.

**Triangle Testing at Maximum Concentration is a Sensitive Predictor of Galaxolide Anosmia on Threshold Testing**

If a subject can perceive Galaxolide at the maximum concentration (dilution step 1 - 50%) they will have a threshold many times lower than that, at least 2.5%, if not less. In any autosomal recessive inheritance pattern, some of these low threshold subjects are very likely to have at least one copy of the non-functional gene. In a dominant pattern, they will have two functional genes. In either case the absolute anosmia is easily detected without using the time-consuming and labour-intensive threshold tests. Therefore, it is feasible to

use just a robust test (multiple 2- or 3-AFC, R-index) at a high concentration (50%) to detect further anosmics, which we then went on to do for the extended family of family three.

**Genotyping: Single Gene Analysis with PCR and Sanger Sequencing**

Although I found a known homozygous SNP (dbSNP: rs7941190) for OR5AN1 in some family members, the variant did not segregate with the phenotype for Galaxolide, Exaltone, habanolide, or musk ketone.

Additionally, the polymorphism is in the predicted cytosolic tail of the receptor and therefore unlikely to alter the binding pocket dynamics. Although as this part of the receptor is thought to be involved with G-Protein interaction there could be an effect with activation of the $G_{olf}$.

The Exac browser (Lek et al. 2016) summarises this as a missense mutation, which is probably damaging, although there are no known clinical associations in ClinVar (Landrum et al. 2016). The SIFT algorithm (Kumar et al. 2009) also classifies the mutation as deleterious. The PolyPhen-2 algorithm (Adzhubei et al. 2010) also predicts the mutation to be probably damaging with a score of 1.000 (sensitivity: 0.00; specificity: 1.00). (PolyPhen-2 v2.2.2r398 HUMDIV model accessed 2017-05-16)

Within the genomes available to the EXaC project, the average allele frequency in almost all ethnic groups is about 0.7%, with a homozygosity rate at least ten times as high in the European (non-Finnish) population than in any of the others.

Although OR5AN1 has been shown to be *a* musk receptor,(Shirasu et al. 2014) detecting muscone, it does not correlate with the distribution of the Galaxolide anosmia phenotype; it is not *the* musk receptor. Either there is at least one other receptor which is pseudogenised or the phenotype is not the result of a receptor variant.

Examining the remaining ~400 ORs by this method would be very labour intensive, but there are more modern techniques enabling an examination of the whole of the olfactory receptor corpus by an exome-wide technique.

**Genotyping: Olfactory Exome Sequencing for OR Variants Accounting for Galaxolide Anosmia**

Any gene with a stop codon or frameshift mutation will not result in a viable protein product. This can be seen in the fact that both of these nonsense mutations OR9K2 and OR6C74 have accumulated several other missense mutations: as pseudogenes, they are not under selection pressure. The same is probably true for OR4K1, which also has four missense mutations. Or2A2 only has two missense SNPs (single nucleotide polymorphisms). This haplotype has a population frequency of 13.82% in the thousand genomes project (Olender et al. 2012) according to the HORDE (http://genome.weizmann.ac.il/horde/card/index/symbol:OR2A2/term:OR2a2/type:keyword) olfactory receptor database and has a CORP pseudogene functionality score of 0.013 (Menashe et al. 2006), indicating that it is probably functional. There are several other heterozygous ORs with only one SNP, which are unlikely to have a functional impact.

All of these variants, apart from OR8U1, probably occur on only one allele in both the anosmics and therefore are unlikely to be the source of a receptor STV.

The obvious OR gene of interest is OR6B3, which is homozygous in both the anosmics and not in the osmics (1.02, 1.04 and 1.06). This is exactly the pattern we would expect to see in a receptor STV: a non-functional gene in both alleles present only in the phenotypically affected group.

There is possibly a single olfactory receptor variant that may account for the phenotypic variation in Galaxolide thresholds: OR6B3. Within the receptor exome and a

small group of genes known to affect olfactory function, there does not seem to be an obvious other candidate which could be the cause of the STV.

OR6B3 has not been a gene of specific interest in the literature. A search with Google Scholar (www.scholar.google.com) for OR6B3, HsOR2.4.2 or OR2-2 (names under old and new nomenclature) shows that it has been used as a marker gene to demonstrate asynchronous transcription of mono allelic gene (Ensminger & Chess 2004) and was one of a group of olfactory receptor genes found to be highly expressed in human trigeminal and dorsal root ganglia in a recent transcriptional study (Flegel et al. 2015). There are no known ligands in the public domain (Flegel et al. 2015). The entry in the HORDE (Glusman et al. 2001) database is similarly barren.

This specific SNP results in an alteration of threonine to isoleucine (T48I) and has not been described before. The ExAC browser (Lek et al. 2016) describes a synonymous SNP at this position but no missense variant, although the canonical transcript coverage is low over this region (approximately 15). The PolyPhen-2 (Adzhubei et al. 2010) protein function prediction algorithm classifies the change as being possibly damaging with a score of 0.952 (sensitivity: 0.79; specificity: 0.95) (PolyPhen-2 v2.2.2r398 HUMDIV model accessed 2017-05-16).

Although this is definitely a gene of interest and will be further investigated as discussed below, two facts make its role in the specific anosmia debatable. The first is the fact that 01/01 is the son of 01/02, who has no copies of the variant gene on this sequencing. It is possible that both of the homozygous alleles arose through uniparental disomy or *de novo* mutation, although provides some caution to the interpretation. The second, related to this, is that the depth of sequence was low for this gene, the lowest in the analysis. The number of reads across this area is low and therefore the accuracy of the variant call is less

than it would be for some of the others (Sims 2014). Of course, this also applies to the sequencing in 1.02, who may have the variant in one allele after all.

**Genotyping: Olfactory Exome Sequencing Gene Variants in Galaxolide Osmic Subjects**

Two interesting gene variants were demonstrated in the Galaxolide osmic subjects: RTP2 and the olfactory receptor OR5AN1.

RTP2

RTP2: Receptor (chemosensory) Transporter Protein 2 is a non-OR gene (https://varsome.com/gene/RTP2) on chromosome 3, an expression factor vital to the trafficking of certain receptors in the heterologous expression systems as discussed in the *in vitro* section of the literature review. Its role *in vivo* seems to be similar although the published literature is sparse (Bush & Hall 2008). Once more the sequence depth has the lowest coverage in the sequencing, with a corresponding caveat to interpretation. The mutation is G100S arising from a C/T exchange at position 3:187416666, but the functional consequence of replacing a glycine with a serine is not known although the variant is described and annotated as 'probably damaging' in the BioMuta database (Hudson et al. 2010). However, the Exac protein browser (Lek et al. 2016) classifies this SNP (dbSNP rs11927120) as a missense variant which is benign and tolerated according to the SIFT algorithm (Kumar et al. 2009). There are 2332 known homozygotes for this polymorphism within the database population, and therefore it is unlikely to be a lethal variant. The clinical genetic database ClinVar (Landrum et al. 2016) lists no known clinical phenotype associated with this variant.

Because the expression pattern of these transport proteins is not known, the heterozygous nature of the mutation may be more significant than for the ORs. Epigenetic factors (Berger et al. 2009) could result in the variant pseudogene being expressed more

often, for instance, even though there is a 'normal' copy at the other site, thus compromising the expression of the GCPRs it traffics.

It is not known which of the ORs require RTP2 to be trafficked. Early expression systems were able to express some ORs without it (McClintock & Sammeta 2003), but the addition of the protein made the expression of a vast majority (but not all) of ORs in a heterologous system more successful (Zhuang & Matsunami 2007),(Saito et al. 2004).

Altering the membrane trafficking of certain receptors could well result in a specific threshold shift phenotype. The variation might improve the function of the RTP2 protein, but is equally likely to result in a loss of function and therefore a specific anosmia would be expected as the receptors were not trafficked to the membrane successfully. Its presence in the Galaxolide detecting group genome suggested that it is unlikely to have a decrease in function, but could be the necessary variant for the successful membrane expression of a functional OR.

OR5AN1

The L289F variant of OR5AN1 is discussed above. It is unlikely to have an effect on the ligand specificity of the olfactory receptor, but may have another effect, such as on the expression levels. It is striking that in this analysis the homozygotes are both anosmic for Galaxolide and the heterozygotes can all smell the molecule. The single gene analysis, which goes on to examine a second family not available to the whole exome analysis, does not agree with this. In the second family, an L/L homozygote is anosmic and another is osmic, whereas an L/L homozygote in the first family is osmic and an F/F homozygote is anosmic. Unfortunately, the overlap in sequencing is too small to compare many of the volunteers in this way but does throw doubt on the role of OR5AN1 in the detection of all musks.

This supports the hypothesis that there is more than one 'musk receptor', although it suggests the subsequent question: if there is more than one musk receptor, do the molecules which stimulate both have such a similar olfactory percept? What is the reason that perfumers call this group of structurally diverse molecules all musks? It is also interesting that the overlap in the STVs for the musks does not divide along structural lines: musk ketone and habanolide (a macrocycle) are structurally very dissimilar and yet share the same STV population distribution (admittedly in a small sample), whereas another macrocyclic musk, Galaxolide, does not.

Once again this may be a result of mucosal/ mucus layer metabolism, with the actual musk odour percept arising not from the molecules themselves, but a common metabolite or group of metabolites, stimulating a different receptor which results in the 'musk' percept. There is no reason why one receptor should not give rise to the same percept as another, or even that the two receptor neurons should not 'wire' to the same glomerulus, and therefore give rise to the same odour sensation, although these situations have never been demonstrated.

## Further Work

To further investigate the vibrational hypothesis in human olfaction, several strands of experiment suggest themselves. For instance, one could improve and extend the chemical and psychophysical investigations with more probe molecules and better techniques, strengthen the genotyping of the STVs, and examine the identified receptors more closely, in a heterologous expression system.

### Psychophysics

To continue the psychophysical investigations of this project would entail improving the techniques to shown robust findings with greater efficiency. One example is the use of a trio test. The comparison of just two samples in each trial requires a large number of

attempts to demonstrate a detectable odour difference. The addition of only one additional comparator sample, a trio test, makes each trial much more powerful. The apparent simplicity of the duo comparison is not outweighed by the need for a large number of trials to achieve significance.

The acetophenone trials were too underpowered to show even the strongest effect. Recruiting more volunteers for a smaller number of trials would have been a more efficient method of demonstrating the effect, or lack thereof, of deuteration. Any further experiments should take that into account.

Although I demonstrated an effect of deuterating musks on their odour character, it may be that this is the only class of odorant compounds which are altered by deuteration. To demonstrate that this is a wider effect, other odorants should be examined. This includes the important case of the negative control: C13 isoptomers. Block et al(Block, Jang, Matsunami, Sekharan, et al. 2015) demonstrated no alteration of activity for olfactory receptors in a heterologous expression system with C13 isoptomers. That these isoptomers do not have altered odour to humans should be confirmed.

The effect of partial deuteration on odour character was shown in an unblinded fashion but is an important finding. Using the same psychophysical techniques as demonstrated in these methods, it would be possible to examine the effect of partial deuteration objectively.

The obvious character of the Galaxolide anosmia, which requires only a simple trio test to determine, makes wider data collection possible. Such a simple test in such a common phenotype (30% according to Wysocki and Gilbert (Wysocki & Gilbert 1989)) allows easy population screening to make the associations of phenotype and genotype more robust. The

more subjects and subject families recruited, the greater the power to detect significant causative changes.

The partial overlap of Galaxolide with musk ketone and habanolide anosmias may be methodological in the small samples presented in this project but a larger population sample may help to determine whether this is a real phenomenon, and its cause.

**Musk receptor**

The common pattern to establish a human receptor-level STV is to identify the phenotype, find the gene of interest and genotype the subjects, correlate the genotype with the phenotype, and then if all of those steps are successful, confirm the *in vitro* activity of the receptor of interest.

More work remains to be done to identify the source of the Galaxolide anosmia. Much of this will rely on the further sequencing and analysis of the collected genomic samples by my colleagues at the Wellcome Trust Sanger Institute and Monell Chemical Senses Institute. If this is not possible then I will approach the 100k genome project (https://www.genomicsengland.co.uk/the-100000-genomes-project/) for assistance.

**Single Gene Analysis**

To investigate the role of OR6B3 we will be confirming the genotype in all available samples with PCR Sanger sequencing. This will be a simple test of the role of the receptor. If it is also a homozygous variant in 1/07 another confirmed Galaxolide anosmic in family 1, then it is more likely to be significant. If the same pattern is confirmed in families 2 and 3, then the finding is very robust.

**Complete Sequencing**

With this experiment, we have identified several genomic loci of interest that segregate with the Galaxolide anosmia phenotype. To increase the power and reliability of

the analysis, the next steps are to include the sequences of the second tranche of DNA samples. We will then re-perform the exome analysis to confirm our findings and possibly identify further genes of interest. This will be cross-referenced with the ligand activity database held by my collaborators at Monell to narrow down the possible candidates.

Once another human musk receptor is identified, further work may include examining homology relationship and functional difference between the receptors as well as their likely evolutionary path. Research questions include why the Galaxolide anosmia is so much more common than that of OR5AN1, and why they share such a similar odour if they activate such different receptors.

**Whole Exome Analysis**

If no receptors are identified, we will then widen the range of the search to all of the possible protein-coding genes - the whole exome. This will vastly increase the number of segregations, merely by the law of large numbers. Although most will not be significant, using the functional database we may be able to narrow the field. This may identify other proteins similar to RTP2, or even confirm the role of the RTP2 heterozygote. We will also cross-reference the olfactory mucus proteins described in (Schilling & Schilling 2009) and (Hélène Débat et al. 2007) to examine the possibility of a peri-receptor event being the key function.

**In-Vitro Expression**

Once a likely set of receptors has been identified, my colleagues at the Monell Chemical Senses Centre in Philadelphia have agreed to express these, likely in a HANA3/HEK 295 dual luciferase reporter system and confirm their *in vitro* activity against a wide range of musks. Although Block et al (Block, Jang, Matsunami, Sekharan, et al. 2015) did not show a variation in OR5AN1 activity with isotopic Exaltone or muscone, we will also examine the activity for isotopic variants of the Galaxolide odorants. Further examination of the

activity of the receptor against a wide musk panel may help to investigate the question of the conserved nature of the musk odour in two separate receptors - why musk odorants share a characteristic scent when they are activated different receptors.

OR6B3 is obviously the receptor most of this work will focus upon, as it has not been characterised in the literature thus far. Any ligands identified should be compared to the activity of their deuterated counterparts if possible.

**Other STVs**

In those families where other musk odorants, musk ketone, and habanolide were examined, the anosmias overlapped with the Galaxolide anosmia with one exception (subject 1/02, who initially reported a Galaxolide anosmia in the yes/no testing too). Further threshold test phenotyping as well as an examination of the relationship once the putative receptors are expressed in a heterologous expression system will demonstrate whether this is due to one receptor variation, or coincidence. If the same receptor is responsible, the different ligands (nitro and macrocyclic) are excellent templates to examine the rules for ligand selectivity in these receptors.

An Exaltone STV has not been demonstrated conclusively in any of the subjects tested so far. Although one has shown a significantly raised threshold, unfortunately the genotype is not available yet. Further recruitment to identify the phenotype of an OR5AN1 pseudogene anosmia would confirm or refute the role of that receptor in the human perception of Exaltone.

**Further work: Clinical Aspects**

Although this thesis only offers some support for the vibrational hypothesis, the theory has much wider application if true. As discussed, the olfactory receptors are members of a much wider family of receptors, the G-protein coupled receptors. These GCPRs are

estimated to be the target for 30% (Beck Sickinger & Budisa 2012) to 60% (Sell 2014) of current pharmacological agents, and if there is a molecular vibration detection mechanism present in one family, why not more? There is now some interest in this effect outside of the olfactory receptor, with theoretical work on some neuroreceptors (Hoehn et al. 2015), although the predictions from that modelling have not withstood experimental investigation (Hoehn et al. 2017).

# Chapter Five: Conclusion

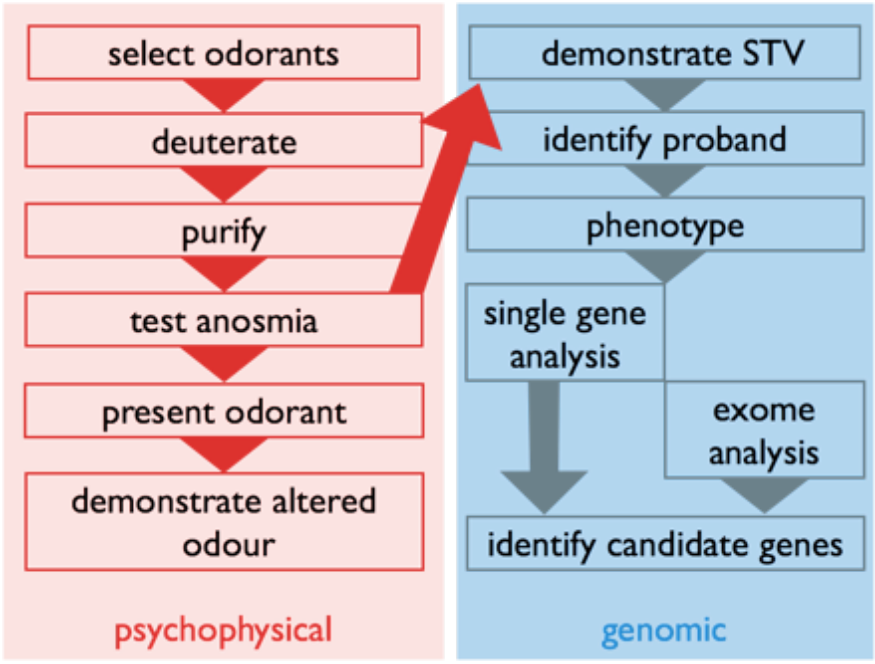



*Figure 110: Outline of project, all stages completed*

In previous chapter I have examined the findings reported in this thesis. I discussed the usefulness of the pilot experiment and the fact that deuteration does not affect the odour character of Acetophenone. I have outlined the possible reasons for this both with and without the assumption of the vibrational hypothesis.

The deuteration of Exaltone does change its odour to humans, and I have discussed the reasons why this might be, again holding the validity of the vibrational hypothesis neutral, with special attention to the fact that this does not seem to be true for acetophenone.

In the search for the receptor which may be responsible for the detection of musks I have phenotyped three ethnically Greek families and shown that the specific anosmia for some musks is likely to be an autosomal recessive mutation with a high population

incidence, causing a pseudo-dominant Mendelian inheritance pattern. I have also demonstrated there is likely to be more than one receptor responsible for the detection of musks.

I have shown that a simple trio test is robust in predicting a specific anosmia for Galaxolide and unfortunately that Exaltone does not have a common specific anosmia.

Finally, and most importantly for further examination of the musk STV and alteration of vibrational characteristics, I have identified a potential new musk receptor: OR6B3.

As to the validity or not of the vibrational hypothesis, I remain in equipoise, although if anything, increasingly the body of evidence does not support it. The most striking finding of this thesis, the alteration of musk odour on deuteration, can be explained by a number of factors other than that of molecular vibration detection and if that is the case then the remaining objective experimental evidence is slim.

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# Appendix A: Search strategy for Literature review

To review the literature, I initially queried the ISI web of Knowledge in 2oo8 with the search strategy as below. The resulting abstracts were then reviewed and all the pertinent results in English read. The relevant sources cited within were retrieved and read. I was also guided to pertinent articles in the literature by my supervisors and colleagues. I performed similar wide searches in 2011, 2014 and 2015.

ISI web of knowledge

1. terms "olfaction" OR "olfact*"
2. "receptor" OR "OR"
3. "olfaction" AND "vibration" AND "vibrational hypothesis"
4. #1 OR #2 AND #3

Pubmed

1. “Olfaction” [Mesh] AND "smell"[MeSH Terms] OR olfaction [Text Word]
2. "receptors, odorant"[MeSH Terms] OR olfactory receptor [ Text Word]
3. Vibration* [ti] OR phonon [ti] OR tunelling [ti] OR quantum [ti]
4. #1 OR #2 AND #3

I set up a Google citation alert with the below terms to capture newly published work as it was published.

1. "human olfactory receptor"
2. human "olfactory receptor"
3. smell, olfaction

# Appendix B: Nomenclature of Olfactory Receptors

There are two alternate systems for naming individual olfactory receptors, the first based on the genetic location and the more recent system based on the sequence homology.

In the older nomenclature, the name starts with a lower-case letter identifying the species (h- for human, m- for mouse etc.), then "OR" to identify it as an olfactory receptor. This is followed by a further two numbers, the chromosome and position on that chromosome. Thus, the receptor hOR-23 is a human receptor occurring on the 23$^{rd}$ chromosome. Occasionally the number is replaced by a significant ligand, e.g. mOR-EG, the mouse receptor which responds to eugenol.

The more recent system, used by the Human Genome Project for identifying the encoding genes for the receptors, identifies the receptor by its homology across species and the name is composed of the letters "OR" and then a family number from 1 to 56. Families are constructed of members sharing more than 40% sequence identity. This is followed by a subfamily letter or two (subfamily members share over 60% sequence identity) and finally another number, identifying the receptor (isoform) uniquely: e.g. OR5AN1 (which can also be called HsOR11.13.3).

# Appendix C: Deorphanising Human Olfactory Receptors

The role of the olfactory receptor is to recognise odorants and any theory of structure-odour relations must take this into account. The vibrational hypothesis is a theory about the mechanism of this recognition step. Knowledge of the ligand specificity of receptors has come a long way since the discovery of the receptors, but only very recently have the techniques described which will allow a large amount of this data to be published in the public domain (It is widely assumed that many of these data are already available to the fragrance companies).

More than half of the non-OR GPCRs have been deorphanised to date (Peterlin et al. 2014) but for the olfactory receptors, almost half of all GPCRs, less than 10% have robustly identified ligands, even in animals. It may be that they have significant difference in molecular function to other GPCRs, but it is true that the technical difficulties have been significant (Peterlin et al. 2014).

The technical strategies used for the 'deorphanisation' of the olfactory receptors can thought of in four broad categories: *in vivo*, *ex vivo*, *in vitro* and *in silico*. Most of these techniques are really only applicable to experimental animals.

Investigating the vibrational hypothesis in human olfaction requires investigation of the structure-odour relationships of the human olfactory receptors. These investigational techniques can be thought of in two phases, before (using specific anosmias) and after, the genomic revolution.

## Specific Threshold Variants (STVs)

As opposed to a total smell-blindness: global anosmia, specific anosmias can be equated to colour-blindness, where the sufferer lacks the ability to detect the odour at all.

Other individual variations in the threshold for an odorant are also useful in the investigation of the olfactory sense. A significantly reduced threshold (specific hyposmia) or raised threshold (hyperosmia) for detection are equally salient phenotypical variants. I propose, and use below, the term "specific threshold variant" (STV) as a catch-all for these. This does not include the more difficult-to-measure variation in valence (pleasantness), perception of odour intensity, or character although these are undoubtedly affected by individual OR variants, as discussed below.

The terms hyp-, hyper- and an-osmia are relative to the performance in perception against a norm. That norm is probably the background prevalence of the functional receptor: if the receptor is common in the population, those not expressing it will be said to have a hyp- or an-osmia. Conversely in a population, most of the members of which have a non-functional receptor, those with a functional receptor will have a hyperosmia. This can obviously vary between populations. The term "STV" captures both of these states without requiring any knowledge of the typical genotype. Historically, however, the literature usually refers to one state or the other, and I have used those terms as appropriate to the papers under discussion.

Specific anosmias have been used to examine the human olfactory system since Amoore pursued them as a "key to the odor code" in the sixties and seventies (Amoore 1967). He developed a "stereochemical theory" (Amoore 1963) attempting to describe all odours by their relations to seven "prime" odours (seven dimensions must have seemed adequate or even generous compared to the three for vision, at least until the description of the real olfactory receptor genome at over 400 functional receptors).

In the pre-genomic era, specific anosmias were described to hydrogen cyanide (Kirk & Stenhouse 1953), n-butyl mercaptan (Patterson & Lauder 1948), isovaleric acid (Griff

& Reed 1995a), β-ionone (Plotto et al. 2006), 1-pyrroline (Amoore et al. 1975) and others. Before the falling cost of genome technology made sequencing possible for individuals, specific anosmias were used to investigate the heritability of olfactory ability in humans (Wysocki & Beauchamp 1984). Although there was some disagreement about the role of heritable factors (Hubert et al. 1980) in specific and general anosmia, in the end, some STVs were demonstrated to be passed down in an autosomal recessive pattern. (Whissell-Buechy 1973) (Wysocki & Beauchamp 1984)

## Deorphanising Human ORs

With the demonstration of a specific anosmia to isovaleric acid in a laboratory mouse (strain C57BL/6J) (Wysocki et al. 1977) came the ability to map the genetic locus responsible: *Iva1*, to chromosome 4 (Griff & Reed 1995b). Using an early mouse reference genome this locus was shown to be a cluster of OR genes (Xinmin Zhang & Firestein 2002) which lent credence to the supposition that this recessive characteristic was receptor dysfunction.

STVs are the natural experiment in human receptor variation required to demonstrate the role of a receptor in human olfaction. In the genomic era Keller et al (Keller et al. 2007) were the first to demonstrate OR7D4 was responsible for a significant part of the human ability to smell androstenone. Not only that but the gene was associated with the valence of the percept: people with non-functional genes were much less likely to report the smell of androstenone as nauseating.

To do this the team expressed 335 putative human olfactory receptors in a HANA3A luciferase reporting system and exposed all of them to a 30 µM solution of aqueous androstenone. They found the OR7D4 receptor to have a significantly greater response than any other expressed receptor. Having identified the receptor of interest, they searched the

SNP databases for polymorphisms, identifying four haplotypes with population frequencies greater than 1%.

Expressing these in the HANA3 cells demonstrated that there was variation in the dose-response curves of the receptors when exposed to androstenone and androstadienone. The variant they referred to as WM showed almost no luciferase activity, whereas another, rarer variant, S84N was more sensitive to the odorant than the commonest "reference type": RT. This may have been because the S84N receptor was better expressed on the cell surface of the expression system for reasons not explored.

Having demonstrated the threshold variation in vitro, 391 volunteers were screened against 66 odours at high and low concentrations, as well as three control odours. One hundred of the RT homozygotes and all of the other genotypes were invited back for thresholding for androstenone and androstadienone. Not all who were invited back underwent the further testing, only 121 in total were tested for the steroid odour thresholds using a single staircase method with seven reversals. The phenotypes were then segregated by genotype and examined.

The WM variant homozygotes demonstrated a greatly elevated threshold for detecting androstenone and androstadienone compared to the RT homozygotes, although even in that RT/RT (commonest) group 28% were anosmic to the odours i.e. unable to detect any odour even at the highest concentration. The study found that the variants could explain approximately 38% of the phenotypic variation.

This paper laid the pattern for identifying a receptor STV in the era of single gene sequencing: identify a phenotype, identify a likely OR gene, sequence that gene and show correlation between the sequence and the phenotype, then confirm the activity, or lack thereof *in vitro*.

Close on the heels of the Keller paper, Menashe et al (Menashe et al. 2007) published a description of a hyperosmia to isovaleric acid. They examined the correlation between 46 identified SNPs in olfactory receptor genes and odour thresholds in a group of 377 individuals. This found a statistical association with a pseudogenising SNP in the OR11H7 and threshold differences in the detection of the "sweaty" odorant isovaleric acid. The team then validated this *in vitro* in a *Xenopus* oocyte heterologous expression system, with a voltage-clamp readout.

Unlike the Keller et al experiments, the possibility that there may be another receptor for isovaleric acid remains, since there was no analysis across a wide range of receptors. Regression analysis shows that the presence of the functional receptor only accounts for about 8% of the variation in thresholds for isovaleric acid, with the authors unable to account for about 40% of the rest.

The continuing improvement in genomic technologies enabled the next identification of a human ligand-receptor pair to use the whole personal genetic sequence of the participants to screen for possible associations, rather than the targeted examination of just one or a few genes.

The personal genetic testing company 23andMe (www.23andme.co.uk) used an online questionnaire to phenotype the perception of asparagus urine. That there is a genetic component to the excretion (Mitchell et al. 1987) and perception (Lison et al. 1980) of odiferous metabolites of asparagus in the urine of those who have just eaten it is now well accepted. The company used the phenotype to identify a linkage disequilibrium area in the genome which included ten ORs, the most significant associations were SNPs in or around the OR2M7 gene. However, they did not confirm the association with threshold testing or *in vitro*, so this remains a putative receptor. Similarly, they showed a SNP within another OR

block was statistically associated with the perception of the taste of fresh coriander (cilantro) as "soapy". The most likely candidate gene was although once again this was not causally linked by further analysis *in vivo* or *in vitro*.

Jaeger et al (Jaeger et al. 2010) performed a similar study, phenotyping 48 people using the R-index method for their thresholds to cis-3-hexen-1-ol, which has the odour of freshly cut grass. Interestingly there was no specific anosmia to the odour but rather an altered threshold. Using a genome-wide approach Several areas of interest were identified as being associated with altered thresholds including a cluster of olfactory receptor genes on chromosome 6 nearest the SNP rs9295791. The most likely target was the receptor OR2J3, of which they identified several haplotypes. In a follow-up (McRae et al. 2012) the same team all expressed 25 ORs within the coding region identified above in a HANA3/luciferase reporter system, confirming that OR2J3 was the gene responsible for alteration in detection thresholds.

Many of the same team (McRae et al. 2013) then widened the net by looking at a large group of volunteer's response to ten odours and their association with a genome-wide search for co-varying genes. Although several likely sites of variation were identified for the perception of odours such as β-damascenone and 2-heptanone, one odorant, β-ionone, was striking in the clear bimodal distribution of detection thresholds within the population. β-ionone is well known as the distinctive scent of parma violets. This variation was isolated to the OR5A1 gene where the homozygous substitution of one amino acid N183D made the detection of the odour require ten times the concentration of the substance. Once again the Matsunami method of expression in the HANA3/HEK293 system with a luciferase reporter confirmed the *in vitro* activity of the receptor. (Jaeger et al. 2013)

In contrast to all of the other human ORs in Table 26, this receptor has a very powerful effect on the subject's ability to smell the odour, being responsible for 96% of the observed variation in phenotype. This is to all intents and purposes a classical Mendelian trait; the ability to detect and also to enjoy the odour of β-ionone is inherited as an autosomal dominant characteristic (i.e. the specific anosmia to β-ionone is an autosomal recessive trait).

All of the studies to this point relied on single nucleotide polymorphisms (SNP) to identify the alteration in receptor function, but a single base-pair alteration is not the only, or even the most significant, way in which a gene function can be altered, even if it is the easiest to identify by matching to a known gene sequence. Setting aside the epigenetic possibilities, copy number variation (CNV) and coding or splicing insertions and deletions (indels) also result in altered protein function without obviously rendering the gene abnormal. Premature stop codons are easy to recognise in a gene sequence as rendering a receptor non-functional, but the detection of these other abnormalities requires high-resolution techniques which have only become feasible in the last few years.

Hasan et al (Hasin et al. 2008) used a high-resolution microarray to examine the copy-number variation in 25 volunteers from three different ethnic backgrounds. They demonstrated a high number of CNVs: 93 in OR genes and 151 affecting pesudogenes. Following on from this work, they used high-throughput sequencing and a novel computational tool to assess the copy number variation across the olfactory receptor genome in one hundred and fifty individuals. (Waszak et al. 2010) The copy-numbers of OR gene loci varied from zero to nine, with some 14.5% having at least one complete deletion of a gene in one individual. 25% of the group had at least one homozygous deletion of an OR gene, some having up to four such "holes" in their functional receptor repertoire.

The team estimated that the CNVs affected about 15% of the OR content. Using the same technique, they identified some known and forty-nine new SNPs which covered about 20% of the known olfactory receptor genes.

This estimate of the variability of the OR genome was further investigated by Olender et al (Olender et al. 2012). Data mining a set of public and private genomic databases, they found that, for the 413 genes for which at least one individual had an open reading frame version, there were over four thousand full-length polypeptide variants. Each individual possessed approximately six hundred allelic variants of the olfactory receptors, one and a half times the number of genetic loci. Combining SNPs and CNVs gave an average number of thirty-five disrupted alleles in any individual.

Joel Mainland and his colleagues (Mainland, Keller, Li, Zhou, Trimmer, Snyder, Moberly, Adipietro, Liu, Zhuang, Zhan, Lee, Lin & Matsunami 2014a) approached the problem from an *in vitro* perspective, using a high-throughput screen of a odorants in a heterologous expression system. They identified eighteen new odorant ligands as well as confirming other, previously published ones. Using data from the 1000 genomes database they identified variants of the OR genes in their assay with a frequency of over 1% and expressed these in the same system. 63% of the receptors examined in this way had a variant that altered function in some way. Using the phenotype data they had collected for guaiacol perception they examined the role of the four identified haplotypes of the OR10G4 receptor. The variation in the receptor predicted 10.3% of the valence and 15.4% of the perceived intensity of guaiacol in the participants. One haplotype differed from the reference OR by eight amino acids and had no response to guaiacol *in vitro*. The role of each amino acid within this variation was investigated by generating a series of receptors that had a SNP for each in turn, but none of the SNPs had an isolated effect on the function of the gene. Rather

it was the combined effect of all together which rendered the receptor unable to detect the odorant.

Increasing sophistication in experimental technologies was required to find the first five confirmed human OR-ligand pairs. This may explain why there are only five so far, summarised in Table 26 although there are another thirty-five putative receptors which remain to be confirmed in one way or another (Mainland, Keller, Li, Zhou, Trimmer, Snyder, Moberly, Adipietro, Liu, Zhuang, Zhan, Lee, Lin & Matsunami 2014a).

| RECEPTOR | ODOUR | PERCEPT | REFERENCE |
|---|---|---|---|
| OR7D4 | androstenone | intensity, valence, character, threshold | Keller 2007(Keller et al. 2007) |
| OR11H7 | isovaleric acid | detection | Menashe 2007 (Menashe et al. 2007) |
| OR2J3 | cis–3-hexenol | detection | McRae 2012(McRae et al. 2012) |
| OR5A1 | β-ionone | threshold, intensity, valence | Jaeger 2013(Jaeger et al. 2013) |
| OR10G4 | guaiacol | intensity, valence | Mainland 2014(Mainland, Keller, Li, Zhou, Trimmer, Snyder, Moberly, Adipietro, Liu, Zhuang, Zhan, Lee, Lin & Matsunami 2014a) |

*Table 26: Currently identified and validated olfactory receptors and their ligands.*

# Appendix D: full list of gene variants in family 1 which vary in anosmic and osmic family members

| Chromosome | Base Pair nu | Sequence de | SNP location | Effect | functional ch | Gene biotype | Gene name | Impact | MA01.01 | MA01.02 | MA01.03 | MA01.04 | MA01.06 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 11 | 56143977 | 42 | Q293R | NON_SYNONYMOU | MISSENSE | protein_coding | OR8U1 | MODERATE | 0/1 | 0/0 | 1/1 | 0/0 | 0/0 |
| 12 | 55523586 | 73 | I12 | FRAME_SHIFT | NONE | protein_coding | OR9K2 | HIGH | 0/1 | 0/0 | 0/1 | 0/0 | 0/0 |
| 12 | 55523685 | 136 | R45C | NON_SYNONYMOU | MISSENSE | protein_coding | OR9K2 | MODERATE | 0/1 | 0/0 | 0/1 | 0/0 | 0/0 |
| 12 | 55523860 | 139 | E103A | NON_SYNONYMOU | MISSENSE | protein_coding | OR9K2 | MODERATE | 0/1 | 0/0 | 0/1 | 0/0 | 0/0 |
| 12 | 55524172 | 117 | R207H | NON_SYNONYMOU | MISSENSE | protein_coding | OR9K2 | MODERATE | 0/1 | 0/0 | 0/1 | 0/0 | 0/0 |
| 12 | 55641075 | 64 | R2G | NON_SYNONYMOU | MISSENSE | protein_coding | OR6C74 | MODERATE | 0/1 | 0/0 | 0/1 | 0/0 | 0/0 |
| 12 | 55641255 | 139 | R62* | STOP_GAINED | NONSENSE | protein_coding | OR6C74 | HIGH | 0/1 | 0/0 | 0/1 | 0/0 | 0/0 |
| 12 | 55641295 | 124 | Y75C | NON_SYNONYMOU | MISSENSE | protein_coding | OR6C74 | MODERATE | 0/1 | 0/0 | 0/1 | 0/0 | 0/0 |
| 12 | 55641328 | 138 | G86D | NON_SYNONYMOU | MISSENSE | protein_coding | OR6C74 | MODERATE | 0/1 | 0/0 | 0/1 | 0/0 | 0/0 |
| 14 | 20216088 | 101 | P168S | NON_SYNONYMOU | MISSENSE | protein_coding | OR4Q3 | MODERATE | 0/1 | 0/0 | 0/1 | 0/0 | 0/0 |
| 14 | 20296010 | 108 | V135L | NON_SYNONYMOU | MISSENSE | protein_coding | OR4N2 | MODERATE | 0/1 | 0/0 | 0/1 | 0/0 | 0/0 |
| 14 | 20404091 | 166 | R89H | NON_SYNONYMOU | MISSENSE | protein_coding | OR4K1 | MODERATE | 0/1 | 0/0 | 0/1 | 0/0 | 0/0 |
| 14 | 20404177 | 144 | M118V | NON_SYNONYMOU | MISSENSE | protein_coding | OR4K1 | MODERATE | 0/1 | 0/0 | 0/1 | 0/0 | 0/0 |
| 14 | 20404614 | 179 | S263R | NON_SYNONYMOU | MISSENSE | protein_coding | OR4K1 | MODERATE | 0/1 | 0/0 | 0/1 | 0/0 | 0/0 |
| 14 | 20404735 | 183 | R304C | NON_SYNONYMOU | MISSENSE | protein_coding | OR4K1 | MODERATE | 0/1 | 0/0 | 0/1 | 0/0 | 0/0 |
| 14 | 22134170 | 45 | E292K | NON_SYNONYMOU | MISSENSE | protein_coding | OR4E2 | MODERATE | 0/1 | 0/0 | 0/1 | 0/0 | 0/0 |
| 2 | 240985347 | 32 | T48I | NON_SYNONYMOU | MISSENSE | protein_coding | OR6B3 | MODERATE | 1/1 | 0/0 | 1/1 | 0/0 | 0/0 |
| 7 | 132193335 | 144 | F40L | NON_SYNONYMOU | MISSENSE | protein_coding | PLXNA4 | MODERATE | 0/1 | 0/0 | 0/1 | 0/0 | 0/0 |
| 7 | 143792991 | 77 | S264N | NON_SYNONYMOU | MISSENSE | protein_coding | OR2A12 | MODERATE | 0/1 | 0/0 | 0/1 | 0/0 | 0/0 |
| 7 | 143806688 | 83 | Q5K | NON_SYNONYMOU | MISSENSE | protein_coding | OR2A2 | MODERATE | 0/1 | 0/0 | 0/1 | 0/0 | 0/0 |
| 7 | 143807304 | 126 | L210P | NON_SYNONYMOU | MISSENSE | protein_coding | OR2A2 | MODERATE | 0/1 | 0/0 | 0/1 | 0/0 | 0/0 |
| 1 | 158735740 | 60 | F245L | NON_SYNONYMOU | MISSENSE | protein_coding | OR6N1 | MODERATE | 0/0 | 0/1 | 0/0 | 0/1 | 0/1 |
| 11 | 4945196 | 56 | Y125S | NON_SYNONYMOU | MISSENSE | protein_coding | OR51G1 | MODERATE | 0/0 | 0/1 | 0/0 | 0/1 | 0/1 |
| 11 | 59132798 | 73 | L289F | NON_SYNONYMOU | MISSENSE | protein_coding | OR5AN1 | MODERATE | 0/0 | 0/1 | 0/0 | 0/1 | 0/1 |
| 11 | 59211188 | 101 | D183N | NON_SYNONYMOU | MISSENSE | protein_coding | OR5A1 | MODERATE | 0/0 | 0/1 | 0/0 | 0/1 | 0/1 |
| 14 | 21109141 | 56 | R237H | NON_SYNONYMOU | MISSENSE | protein_coding | OR6S1 | MODERATE | 0/0 | 0/1 | 0/0 | 0/1 | 0/1 |
| 3 | 187416666 | 32 | G100S | NON_SYNONYMOU | MISSENSE | protein_coding | RTP2 | MODERATE | 0/0 | 0/1 | 0/0 | 0/1 | 0/1 |
| 6 | 29364615 | 74 | V47F | NON_SYNONYMOU | MISSENSE | protein_coding | OR12D2 | MODERATE | 0/0 | 0/1 | 0/0 | 0/1 | 0/1 |
| 6 | 29364643 | 100 | L56P | NON_SYNONYMOU | MISSENSE | protein_coding | OR12D2 | MODERATE | 0/0 | 0/1 | 0/0 | 0/1 | 0/1 |
| 6 | 29364815 | 126 | F113L | NON_SYNONYMOU | MISSENSE | protein_coding | OR12D2 | MODERATE | 0/0 | 0/1 | 0/0 | 0/1 | 0/1 |
| 6 | 29364835 | 118 | L120R | NON_SYNONYMOU | MISSENSE | protein_coding | OR12D2 | MODERATE | 0/0 | 0/1 | 0/0 | 0/1 | 0/1 |
| 6 | 29364951 | 70 | V159I | NON_SYNONYMOU | MISSENSE | protein_coding | OR12D2 | MODERATE | 0/0 | 0/1 | 0/0 | 0/1 | 0/1 |

The list here is presented in several tables in the main text. Each gene is identified by its chromosome number, base pair number, sequence description, and the amino acid replacement (SNP location). Its effect and functional change is reported, as well as its ontology and name. The columns MA are the family members of family one and the zygosity of the SNP is reported in each column.

# Appendix E: Musk receptor groups

| AUTHORS | ASSAY | MUSK GROUPS |
|---|---|---|
| AMOORE (AMOORE ET AL. 1977). | psychophysical | Galaxolide |
| | | Pentadecalone, musk ketone |
| WHISSEL-BUECHY (WHISSELL-BUECHY 1973) | psychophysical | Pentadecalone, musk ambrette |
| | | Musk ketone |
| GILBERT (GILBERT & KEMP 1996), | psychophysical | Cyclopentadecanone and musk xylol |
| | | Ambrettolide, tonalid |
| NARA (NARA ET AL. 2011) | mouse receptor ex vivo | Hexadecanolide, musk ambrette, ambrettone, muscone |
| | | Musk ambrette |
| | | Civettone |
| SHIRASU (SHIRASU ET AL. 2014) | mouse receptor in vivo | Muscone, ambrettone, cyclopentadecanone |
| | | Exaltolide, ethylene brassylate, musk xylol, musk ketone, tonalid, galaxolide |
| MCCLINTOCK (MCCLINTOCK ET AL. 2014) | mouse receptor in vitro | Muscone |
| | | Tonalid, galaxolide, Astrotone |

*Table 27: Musks fall into at least two groups, with at least two different receptors*

# Appendix F: R markdown scripts

## psychophysics results

```
#script for the analysis of acetophenone data
# import data fomr csv as a data table
DT <- as.data.table(read.csv(file = file.choose()))
# check it has a session column
is.na(DT$session)
#check the structure of the DT
str(DT)
# clean up and reorder
DT <- DT[,c(12,11,2,4:7,3,9,10)]
#standardise the names
names(DT) <- c("volunteer", "session", "trialnumber",
"firstsampleID","secondsampleID","firstsamplevalue","secondsamplevalue","answer","correct")
# give it a comparison column
DT$comparison <-
as.factor(ordered(ifelse(DT[,firstsamplevalue]=="D"&DT[,secondsamplevalue]=="D","DvD",
                         ifelse(DT[,firstsamplevalue]=="H"& DT[,secondsamplevalue]=="D","HvD",
                         ifelse(DT[,firstsamplevalue] =="D" & DT[,secondsamplevalue]=="H",
"DvH","HvH"))),levels = c("HvD","DvH","DvD","HvH")))


# columns as factors
DT$session <- as.factor(DT$session)

#save the cleaned results
write.csv(DT, file = paste0(levels(DT$volunteer),".resultsformatted.csv", sep =""))
#produce a summary table and calcuate the binomial statistics
DT.sessionpair <- binomilise(DT[,.(success = sum(correct),trials = .N), by= .(session,comparison)])
DT.session <- binomilise (DT[,.(success = sum(correct), trials = .N), by = session])
DT.half <- binomilise(DT[,.(success= sum(correct), trials = .N), by = .(halves = ifelse(trialnumber %in%
1:(nrow(DT)/2),"first half", "second half"))])
DT.pair <- binomilise(DT[,.(success = sum(correct), trials = .N), by = comparison])
DT.samediff <- binomilise(DT[,.(success = sum(correct), trials = .N), by = .(samedifferent =
ifelse(comparison =="HvH"|comparison =="DvD","same","different"))])


#kable(DT.sessionpair)
if (nlevels(DT$session)>1) {kable(DT.session)}
if (nlevels(DT$session)<1) {kable(DT.session[,c(2:7)])}
if (nlevels(DT$session)>1) {kable(DT.sessionpair)}
if (nlevels(DT$session)<1) {kable(DT.half)}
kable(DT.samediff)
kable(DT.pair)
DT.all <-  paste0(c(kable(DT.session),kable(DT.half),kable(DT.pair),kable(DT.samediff),
kable(DT.sessionpair)), sep = "", collapse = "")
```

```
#plot the results
sessionpairplot <- ggplot(DT.sessionpair, aes(x = session,y = estimate,ymax = upperCI,ymin = lowerCI,
colour = comparison, show.legend = FALSE, group = comparison)) +
 geom_point(size = 5, position = position_dodge(width = 0.5))+
 geom_errorbar(width = 0.25,alpha = .75,show.legend = FALSE, position = position_dodge(width =
0.5))+
 geom_line(position = position_dodge(width = 0.5), alpha = 0.2) +
 geom_abline(slope = 0,intercept = 50, linetype = "dashed", colour ="red")+
 xlab("session") +
 ylab("%Successes") +
 theme_bw()
ggsave(paste0("/Users/simonbcgane/Dropbox/PhD/Results/Psychophysics/Acetophenone
analysis/",levels(DT$volunteer)," results/",levels(DT$volunteer),"sessionpairplot.pdf", sep = ""),
width= 8.27, height=11.69, dpi=300)
#
sessionplot <- ggplot(DT.session, aes(x = session,y = estimate,ymax = upperCI,ymin = lowerCI, colour
= session, show.legend = FALSE, group = session)) +
 geom_point(size = 5, position = position_dodge(width = 0.5))+
 geom_errorbar(width = 0.25,alpha = .75,show.legend = FALSE, position = position_dodge(width =
0.5))+
 geom_abline(slope = 0,intercept = 50, linetype = "dashed", colour ="red")+
 xlab("session") +
 ylab("%Successes") +
 theme_bw()
ggsave(paste0("/Users/simonbcgane/Dropbox/PhD/Results/Psychophysics/Acetophenone
analysis/",levels(DT$volunteer)," results/",levels(DT$volunteer),"sessionplot.pdf", sep = ""), width=
8.27, height=11.69, dpi=300)
#
pairplot <- ggplot(DT.pair, aes(x = comparison,y = estimate,ymax = upperCI,ymin = lowerCI, colour =
comparison, show.legend = FALSE, group = comparison)) +
 geom_point(size = 5, position = position_dodge(width = 0.5))+
 geom_errorbar(width = 0.25,alpha = .75,show.legend = FALSE, position = position_dodge(width =
0.5))+
 geom_abline(slope = 0,intercept = 50, linetype = "dashed", colour ="red")+
 xlab("session") +
 ylab("%Successes") +
 theme_bw()
ggsave(paste0("/Users/simonbcgane/Dropbox/PhD/Results/Psychophysics/Acetophenone
analysis/",levels(DT$volunteer)," results/",levels(DT$volunteer),"pairplot.pdf", sep = ""), width=
8.27, height=11.69, dpi=300)
#
halfplot <- ggplot(DT.half, aes(x = halves,y = estimate,ymax = upperCI,ymin = lowerCI, colour =
halves, show.legend = FALSE, group = halves)) +
 geom_point(size = 5, position = position_dodge(width = 0.5))+
 geom_errorbar(width = 0.25,alpha = .75,show.legend = FALSE, position = position_dodge(width =
0.5))+
 geom_line(position = position_dodge(width = 0.5), alpha = 0.2) +
 geom_abline(slope = 0,intercept = 50, linetype = "dashed", colour ="red")+
 xlab("session") +
 ylab("%Successes") +
```

```
  theme_bw()
ggsave(paste0("/Users/simonbcgane/Dropbox/PhD/Results/Psychophysics/Acetophenone
analysis/",levels(DT$volunteer)," results/",levels(DT$volunteer),"halfplot.pdf", sep = ""), width=
8.27, height=11.69, dpi=300)
#
samediffplot <- ggplot(DT.samediff, aes(x = samedifferent,y = estimate,ymax = upperCI,ymin =
lowerCI, colour = samedifferent, show.legend = FALSE, group = samedifferent)) +
  geom_point(size = 5, position = position_dodge(width = 0.5))+
  geom_errorbar(width = 0.25,alpha = .75,show.legend = FALSE, position = position_dodge(width =
0.5))+
  geom_line(position = position_dodge(width = 0.5), alpha = 0.2) +
  geom_abline(slope = 0,intercept = 50, linetype = "dashed", colour ="red")+
  xlab("session") +
  ylab("%Successes") +
  theme_bw()
ggsave(paste0("/Users/simonbcgane/Dropbox/PhD/Results/Psychophysics/Acetophenone
analysis/",levels(DT$volunteer)," results/",levels(DT$volunteer),"samediffplot.pdf", sep = ""), width=
8.27, height=11.69, dpi=300)
#

multiDT <- multiplot(pairplot, sessionplot,sessionpairplot, layout =  matrix(c(1,2,3,3), nrow=2,
byrow=TRUE))
ggsave(paste0("/Users/simonbcgane/Dropbox/PhD/Results/Psychophysics/Acetophenone
analysis/",levels(DT$volunteer)," results/",levels(DT$volunteer),"multiplot.pdf", sep = ""), width=
8.27, height=11.69, dpi=300)
#pdf(file = paste0("/Users/simonbcgane/Dropbox/PhD/Results/Psychophysics/Acetophenone
analysis/",levels(DT$volunteer)," results/",levels(DT$volunteer),"multiplot.pdf", sep = ""), paper =
"a4r")


DT[, .(success= sum(correct), trials = .N), by = .(trialnumber %in% 1:(nrow(DT)/2))]
write.csv(DT, file =
paste0("/Users/simonbcgane/Dropbox/PhD/Results/Psychophysics/Acetophenone
analysis/",levels(DT$volunteer)," results/",levels(DT$volunteer),"datatable.csv", sep = ""))
write.csv(DT.pair, file =
paste0("/Users/simonbcgane/Dropbox/PhD/Results/Psychophysics/Acetophenone
analysis/",levels(DT$volunteer)," results/",levels(DT$volunteer),"pairdatatable.csv", sep = ""))
write.csv(DT.session, file =
paste0("/Users/simonbcgane/Dropbox/PhD/Results/Psychophysics/Acetophenone
analysis/",levels(DT$volunteer)," results/",levels(DT$volunteer),"sessiondatatable.csv", sep = ""))
write.csv(DT.sessionpair, file =
paste0("/Users/simonbcgane/Dropbox/PhD/Results/Psychophysics/Acetophenone
analysis/",levels(DT$volunteer)," results/",levels(DT$volunteer),"datatable.csv", sep = ""))
```

## Musk

```
---
title: "musk anosmia psychophysics"
author: "Simon Gane"
date: "01/03/2017"
output: word_document
classoption: landscape
---
```r global_options, include=FALSE}
knitr::opts_chunk$set(fig.path='Figs/',
                      echo=FALSE, warning=FALSE, message=FALSE, dpi=300, echo=F, comment="")
```
```r setup, include=FALSE}

library(ggplot2)
library(data.table)
library(RColorBrewer)
library(knitr)
library(Hmisc)

```

```r get Musk results}
MR <- as.data.table(read.csv(file = "all musk phenotype.csv", na.strings = NA, row.names = NULL))
#MR$Gal.rank <- as.factor(MR$Gal.rank)
MR$Gal.dil.step <- as.factor(MR$Gal.dil.step)
#MR$Exa.rank <- as.factor(MR$Exa.rank)
MR$DNA.tranche <- as.factor(MR$DNA.tranche)
MR$family <- as.factor(MR$family)


#make a table
MRprint <- MR
colnames(MRprint) <- gsub("[.]", " ",names(MRprint))
kable(MRprint[,2:16])
```

## Does the ability to detect galaxolide correlate with the ability to detect Exaltone?
```r spearman correlation, echo=FALSE}
cor.test(MR$Gal.rank,MR$Exa.rank, method ="spearman")

```
##Plot of galaxolide vs Exaltone threshold rank

```r smoking}
ggplot(MRpri, aes(x = Exa dil step, y = Gal dil step))+
  geom_point(aes(shape = smoker, color=smoker, size = 10))
```
```

# Appendix G: Gene list

A .bed file is available with the list of genes, locations and intervals.

| | | | | | |
|---|---|---|---|---|---|
| OR14C36 | OR2L5 | RAB6B | ZKSCAN5 | TUBB2C | OR52L1 |
| OR4F5 | OR2L2 | CLSTN2 | OR2AE1 | OR13H1 | OR56A4 |
| OR4F29 | OR2L3 | PCOLCE2 | GPR85 | TMSB4X | OR56A1 |
| OR4F3 | OR2M5 | MBNL1 | IMPDH1 | TMSL3 | OR56B4 |
| OR4F16 | OR2M2 | MFSD1 | PLXNA4 | TSPAN7 | OR52B2 |
| GNB1 | OR2M3 | CLCN2 | OR9A4 | MMGT1 | OR52W1 |
| SKI | OR2M4 | RTP1 | OR9A2 | CNGA2 | CNGA4 |
| KIF1B | OR2T33 | RTP2 | OR6V1 | GDI1 | OR2AG2 |
| HNRNPR | OR2T12 | CTBP1 | OR10AC1 | DCLRE1C | OR2AG1 |
| ARID1A | OR2M7 | D4S234E | OR2F2 | KIF5B | OR6A2 |
| SDC3 | OR2T4 | PPP2R2C | OR2F1 | OR13A1 | OR10A5 |
| SSX2IP | OR2T6 | KLHL5 | OR6B1 | SGPL1 | OR10A2 |
| BRDT | OR2T1 | RASL11B | OR2A5 | UNC5B | OR10A4 |
| RPL5 | OR2T7 | RUFY3 | OR2A25 | ZMIZ1 | OR2D2 |
| DPH5 | OR2T2 | CCNI | OR2A12 | PTEN | OR2D3 |
| KCNC4 | OR2T3 | RAP1GDS1 | OR2A2 | PGAM1 | OR5P2 |
| ATP1A1 | OR2T5 | RNF150 | OR2A14 | GSTO1 | OR5P3 |
| ATP1A1OS | OR2G6 | FSTL5 | OR2A42 | TOLLIP | OR10A6 |
| IGSF3 | OR2T29 | CPE | OR2A1 | OR52B4 | OR10A3 |

| | | | | | |
|---|---|---|---|---|---|
| HIST2H2BE | OR2T34 | SDHA | OR2A9P | OR52K2 | TUB |
| SYT11 | OR2T10 | NDUFS6 | OR2A20P | OR52K1 | RIC3 |
| OR10T2 | OR2T11 | CTXN3 | LOC728377 | OR52M1 | EIF4G2 |
| OR10K2 | OR2T35 | SLC12A2 | OR2A7 | OR52I2 | CALCA |
| OR10K1 | OR2T27 | KIF3A | OR2A9 | OR52I1 | SAA4 |
| OR10R2 | OR14I1 | AFF4 | OR4F21 | OR51D1 | SAA2-SAA4 |
| OR6Y1 | FZD7 | FAM53C | EBF2 | OR51E1 | SLC17A6 |
| OR6P1 | CENPO | NDFIP1 | DPYSL2 | OR51E2 | SLC1A2 |
| OR10X1 | ADCY3 | ODZ2 | TMEM66 | OR51F1 | TRAF6 |
| OR10Z1 | RASGRP3 | CPLX2 | RBM12B | OR52R1 | CRY2 |
| OR6K2 | CALM2 | OR2Y1 | SPAG1 | OR51F2 | CREB3L1 |
| OR6K3 | RFX8 | OR2V2 | YWHAZ | OR51S1 | MADD |
| OR6K6 | WDSUB1 | GNB2L1 | RAD21 | OR51T1 | OR4B1 |
| OR6N1 | SCN9A | PHACTR1 | KHDRBS3 | OR51A7 | OR4X2 |
| OR6N2 | RBM45 | OR2B2 | VCP | OR51G2 | OR4X1 |
| OR10J3 | NIF3L1 | OR2B6 | OR13J1 | OR51G1 | OR4S1 |
| OR10J4 | IGFBP2 | OR2W1 | OR2S2 | OR51A4 | OR4C3 |
| OR10J1 | FAM134A | LOC100129636 | RFK | OR51A2 | OR4C45 |
| OR10J5 | WDFY1 | OR2B3 | CEP78 | OR51L1 | OR4A47 |
| IGSF8 | SERPINE2 | OR2J3 | TGFBR1 | OR52J3 | OR4C13 |
| PCP4L1 | PTMA | OR2J2 | OR13F1 | OR52E2 | OR4C12 |
| ATP1B1 | OR6B2 | OR14J1 | OR13C4 | OR52A4 | OR4A5 |

| | | | | | |
|---|---|---|---|---|---|
| RABGAP1L | OR6B3 | OR5V1 | OR13C3 | OR52A5 | OR4A8 |
| IVNS1ABP | HDLBP | OR12D3 | OR13C8 | OR52A1 | OR4C46 |
| NAV1 | CNTN4 | OR12D2 | OR13C5 | OR52Z1 | OR4A16 |
| ADIPOR1 | EAF1 | OR12D1 | OR13C2 | OR51V1 | OR4A15 |
| RCOR3 | GOLGA4 | OR11A1 | OR13C9 | OR51B4 | OR4C15 |
| WNT3A | RPSA | OR10C1 | OR13D1 | OR51B2 | OR4C16 |
| OR2B11 | RBM15B | OR2H1 | OR2K2 | OR51B5 | OR4C11 |
| OR2W5 | PPM1M | OR2H2 | STOM | OR51B6 | OR4P4 |
| LOC148824 | TMF1 | GABBR1 | OR1J1 | OR51M1 | OR4S2 |
| OR2C3 | OR5AC1 | FOXP4 | OR1J2 | OR51Q1 | OR4C6 |
| OR2G2 | OR5AC2 | TMEM63B | OR1J4 | OR51I1 | OR5D13 |
| OR2G3 | OR5H1 | HSP90AB1 | OR1N1 | OR51I2 | OR5D14 |
| unknown | OR5H14 | TNFRSF21 | OR1N2 | OR52D1 | OR5L1 |
| OR13G1 | OR5H15 | KLHL31 | OR1L8 | OR52H1 | OR5D18 |
| OR6F1 | OR5H6 | EEF1A1 | OR1Q1 | OR52B6 | OR5L2 |
| OR1C1 | OR5H2 | MYO6 | OR1B1 | OR56B1 | OR5D16 |
| OR14A16 | OR5H8 | OR2A4 | OR1L1 | OR52N4 | OR5W2 |
| OR11L1 | OR5K4 | ENPP3 | OR1L3 | OR52N5 | OR5I1 |
| OR2W3 | OR5K3 | LRP11 | OR1L4 | OR52N1 | OR10AG1 |
| OR2T8 | OR5K1 | SNX9 | OR1L6 | OR52N2 | OR5F1 |
| OR2L13 | OR5K2 | ACTB | OR5C1 | OR52E6 | OR5AS1 |
| OR2L8 | ATP6V1A | GLCCI1 | OR1K1 | OR52E8 | OR8I2 |

| | | | | | |
|---|---|---|---|---|---|
| OR2AK2 | GRAMD1C | PDE1C | STRBP | OR52E4 | OR8H2 |
| OR2L1P | GSK3B | OGDH | LHX2 | OR56A3 | OR8H3 |
| PLXNA1 | OLFM1 | AK1 | OR56A5 | | |

*Table 28: List of genes examined in the olfactory mucosa exome. Several unnamed genes, known to be expressed in mouse olfactory mucosa are represented here by "unknown".*

# Appendix H: Ethical considerations and documents

## Psychophysics

### Ethics Board Application

Ethical approval was obtained from UCL research ethics committee as number 1585/001 "investigating the genetics of human olfaction" (see Figure 112 below) and amended, to change the investigators after the death of my supervisor and to include a salivary sample rather than a blood test. Local ethical approval for Greek subjects was obtained from the Alexander Fleming institute: reference number 3433/16.09.2013. (Figure 113).

**UCL GRADUATE SCHOOL**
UCL RESEARCH ETHICS COMMITTEE

Professor Stoneham
London Centre for Nanotechnology
UCL
17-19 Gordon Street
London
WC1H 0AH

15 July 2008

Dear Professor Stoneham

**Notification of Ethical Approval**
**Project ID/Title: 1585/001: Can humans detect isotopes by smell?**

I am pleased to confirm that in my capacity as Chair of the UCL Research Ethics Committee I have approved your study for a period of 12 months from the commencement of the project (21 July 2008).

Approval is subject to the following conditions:

1. It is a requirement of the Committee that research projects which have received ethical approval are monitored annually. Therefore, you must complete and return our 'Annual Continuing Review Approval Form' PRIOR to the **21 July 2009**. If your project has ceased or was never initiated, it is still important that you complete the form so that we can ensure that our records are updated accordingly.

2. You must seek Chair's approval for proposed amendments to the research for which this approval has been given. Ethical approval is specific to this project and must not be treated as applicable to research of a similar nature. Each research project is reviewed separately and if there are significant changes to the research protocol you should seek confirmation of continued ethical approval by completing the 'Amendment Approval Request Form'.

The forms identified above can be accessed by logging on to the ethics website homepage: http://www.grad.ucl.ac.uk/ethics/ and clicking on the button marked 'Responsibilities Following Approval'.

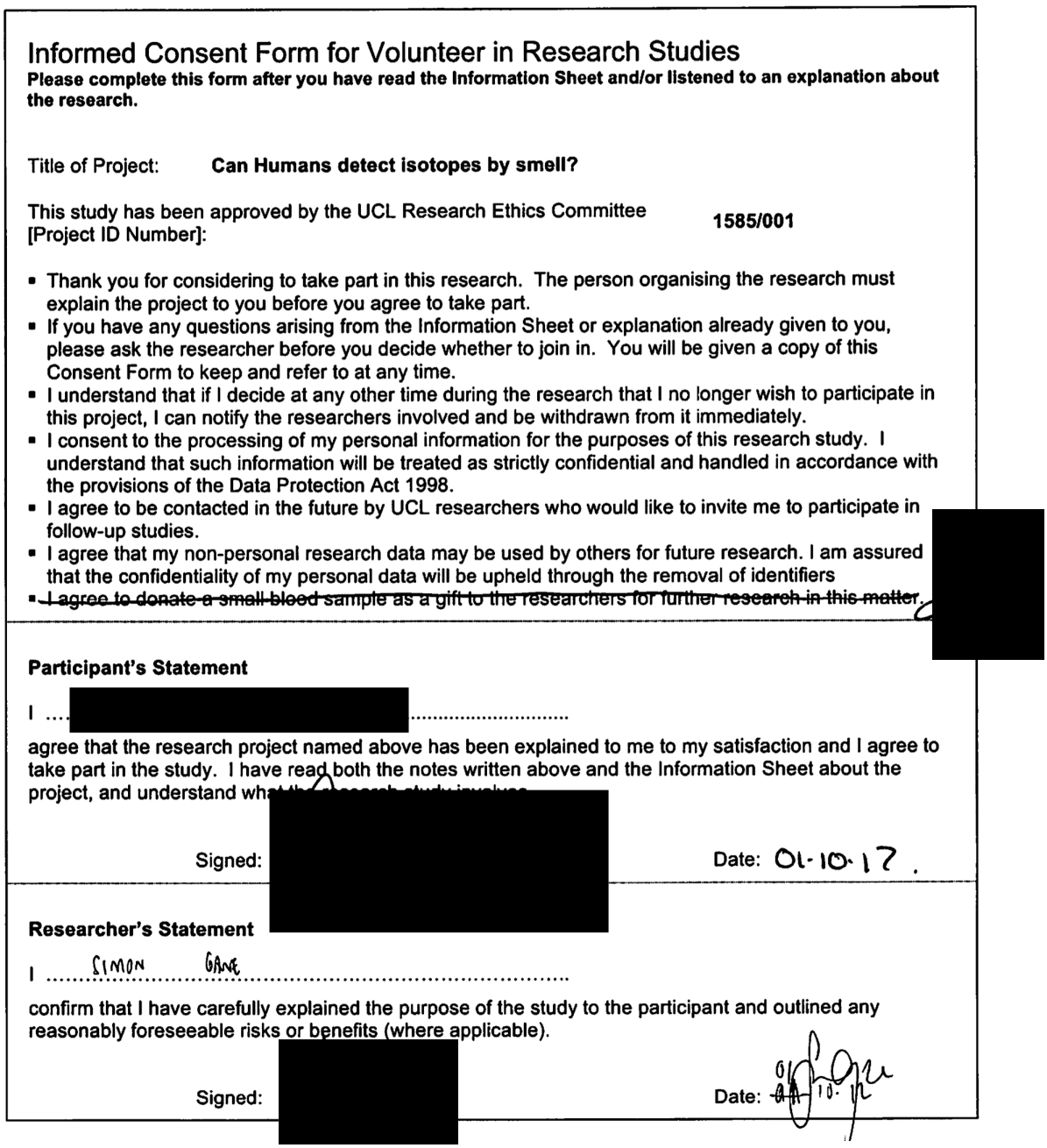
3. It is your responsibility to report to the Committee any unanticipated problems or adverse events involving risks to participants or others. Both non-serious and serious adverse events must be reported.

   **Reporting Non-Serious Adverse Events.**
   For non-serious adverse events you will need to inform Ms Helen Dougal, Ethics Committee Administrator (h.dougal@ucl.ac.uk), within ten days of an adverse incident occurring and provide a full written report that should include any amendments to the participant information sheet and study protocol. The Chair or Vice-Chair of the Ethics Committee will confirm that the incident is non-serious and report to the Committee at the next meeting. The final view of the Committee will be communicated to you.

   **Reporting Serious Adverse Events**
   The Ethics Committee should be notified of all serious adverse events via the Ethics Committee Administrator immediately the incident occurs. Where the adverse incident is unexpected and serious, the Chair or Vice-Chair will decide whether the study should be terminated pending the opinion of an independent expert. The adverse event will be considered at the next Committee meeting and a decision will be made on the need to change the information leaflet and/or study protocol.

*Figure 111: Initial approval for project*

Consent Form



## Informed Consent Form for Volunteer in Research Studies

**Please complete this form after you have read the Information Sheet and/or listened to an explanation about the research.**

Title of Project: **Can Humans detect isotopes by smell?**

This study has been approved by the UCL Research Ethics Committee [Project ID Number]: **1585/001**

- Thank you for considering to take part in this research. The person organising the research must explain the project to you before you agree to take part.
- If you have any questions arising from the Information Sheet or explanation already given to you, please ask the researcher before you decide whether to join in. You will be given a copy of this Consent Form to keep and refer to at any time.
- I understand that if I decide at any other time during the research that I no longer wish to participate in this project, I can notify the researchers involved and be withdrawn from it immediately.
- I consent to the processing of my personal information for the purposes of this research study. I understand that such information will be treated as strictly confidential and handled in accordance with the provisions of the Data Protection Act 1998.
- I agree to be contacted in the future by UCL researchers who would like to invite me to participate in follow-up studies.
- I agree that my non-personal research data may be used by others for future research. I am assured that the confidentiality of my personal data will be upheld through the removal of identifiers
- ~~I agree to donate a small blood sample as a gift to the researchers for further research in this matter.~~

[redacted]

**Participant's Statement**

I ....[redacted].....

agree that the research project named above has been explained to me to my satisfaction and I agree to take part in the study. I have read both the notes written above and the Information Sheet about the project, and understand wh[redacted]

Signed: [redacted] Date: 01.10.17.

**Researcher's Statement**

I ....SIMON GANE.....

confirm that I have carefully explained the purpose of the study to the participant and outlined any reasonably foreseeable risks or benefits (where applicable).

Signed: [redacted] Date: 01.10.17

*Figure 112: Sample consent form for psychophysics experiments*

**Control Information Sheet (Version 6)**

Part 1:

Study Title:

Can Humans detect heavy water by smell alone?

You are being invited to take part in a research study. Before you decide if you wish to take part it is important for you to understand why the research is being done and what it will involve. The following information should help you to make this decision. Please take time to read the information in this sheet carefully and discuss it with friends, relatives and your family doctor if you wish. Please ask us if there is anything that is not clear or if you would like more information (contact numbers and emails at end). Take time to decide if you wish to take part.

Thank you for reading this.

What is the purpose of the study?

The underlying mechanisms of the sense of smell are still not fully understood. There are several competing theories and they disagree whether humans can smell the difference between molecules of the exact same shape but different weights. We will test whether this is the case.

Why have I been invited?

You have been invited because we will perform this test in healthy people with an otherwise normal sense of smell Do I have to take part? It is up to you to decide whether or not to take part. If you do decide to take part you will be asked to sign a consent form. You do not have to If you decide to take part you are still free to withdraw at any time and without giving a reason and you have the right to refuse any of the routine diagnostic tests if you so wish.

What will happen to me if I take part?

We will talk with you about the study and if you are happy to continue we will ask you to sign a consent form saying so. You will be given a copy of the consent form to keep.

We will:

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1. Ask you to complete a nose health questionnaire.

2. Ask you to do a quick normal smell test.

3. Present you with some training test tubes and tell you which smell is which.

4. Present you with some trial samples and ask you to identify which are which.

5. We may do this more than once if you agree.

What are the possible disadvantages and risks of taking part?

The testing will take about an hour.

What are the possible benefits of taking part?

There will be no immediate benefits for you if you agree to help with this study, apart from a complete evaluation of your sense of smell. If you wish, we will be able to tell you the results of some of your tests.

What if there is a problem?

Any complaint about the way you have been dealt with during the study or any possible harm you might suffer will be addressed.

Will my taking part in this study be kept confidential?

All information that is collected about you during the research will be kept strictly confidential and securely. Only the investigators involved in the study will have access to it. Any information about you that leaves the test centre will have your name and address removed so that you cannot be recognised from it. Your identity will be protected when results are published. All the data will be stored in accordance with the Data Protection Act of 1998

If the information in Part 1 has interested you and you are considering participation, please read the additional information in Part 2 before making any decision.

Part 2 of the information sheet:

2.1 What if relevant new information becomes available?

Sometimes we get new information about the phenomenon being studied. If this happens, your researcher will tell you and discuss whether you should continue in the study. If you decide to continue in the study s/he may ask you to sign an updated consent form.

2.2 What will happen if I don't want to carry on with the study?

You can withdraw from treatment but keep in contact with us to let us know your progress. Information collected may still be used.

2.3 What if there is a problem?

Complaints:

If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions (tel: 020 79151674). If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure. Details can be obtained from the hospital.

Harm:

It is extremely unlikely that you will come to any harm as a result of being on this trial as it is an observational study.

2.4 Will my taking part in the study be kept confidential?

All information which is collected about you during the course of the research will be kept strictly confidential, and any information about you which leaves the hospital will have your name and address removed so that you cannot be recognised.

If you join the study, the data collected will be looked at by authorised persons from the team organising the research. They may also be looked at by representatives of regulatory authorities and by authorised people to check that the study is being carried out correctly. All will have a duty of confidentiality to you as a research participant and we will do our best to meet this duty. Data collected during the study may be sent to associated researchers to countries where the laws don't protect your privacy to the same extent as the Data Protection Act in the UK but the data will not contain any identifying information and we will take all reasonable steps to protect your privacy.

2.5 What will happen to any samples I give?

You will provide some new samples of blood. These will be taken by hospital staff and processed by the hospital laboratory or held for further investigation. Some of the blood and tissue samples may be transferred to a Laboratory at the University College London. All samples are not labelled with your name but instead with a study number which links to your questionnaire information but not any identifying data.

2.6 What will happen to the results of the research study?

We hope that the results of the study will be published in a leading scientific journal. After publication we would be pleased to inform you of the results and discuss them with you.

2.7 Who is organising and funding the research?

This study is organised by Dr Simon Gane and Professor Marshall Stoneham of University College London. It is funded by the NanolRC as part of the work towards the completion of Dr Gane's Doctorate degree.

2.8 Who has reviewed the study?

All research at UCL is looked at by independent group of people, called a Research Ethics Committee to protect your safety, rights, wellbeing and dignity. This study has been reviewed and given favourable opinion by the University College Local Research Ethics Committee.

2.9 Further Information and contact details

General information about medical research can be found on the website: http://clinicaltrials.gov/ct/info/resources

For more information about this specific project, please contact Simon Gane on xnumber redactedx or [redacted]. He will also be able to give you advice about taking part in this study. This would also be the first place to contact if you have any concerns about the trial.

Thank you for reading this information.

## Genetics

Local Ethics Approval

"ALEXANDER FLEMING"
Biomedical Sciences Research Center

Local ethics review permission for "musk olfactory receptor family study"

University College London Ethics review number: 1585/001
("Can humans detect heavy water by smell?")

Ref number: 3433/16.09.2013

TO WHOM IT MAY CONCERN

In the light of this study already having ethical approval by the board of a University within the EU (University College London, UK); I hereby provide local ethical permission for the study to take place in Greece as a "chairman's action".

It will be subject to the same guidelines and protocols as laid out in the application for UK ethical review as listed above.

Yours Sincerely,

[redacted]

Babis Savakis
Scientific Director and Chairman of the Board

34, Al. Fleming Str., Vari 166 72, P.O.Box 74145 Varkiza 166 02, HELLAS, tel.: (+30) 210 9656310 -11, fax: (+30) 210 9653934
secretariat@fleming.gr - http://www.fleming.gr

*Figure 113: Local ethical approval for Greece*

**Consent Form**

**Volunteer number: MR...../.....**

**Informed Consent Form for Volunteer in Research Studies**
Please complete this form after you have read the Information Sheet and/or listened to an explanation about the research.

**Title of Project: Musk receptor genetics study**
**UCL Ethics approval number: (part of: Can Humans detect Heavy water in molecules by smell?)1585/001**

Thank you for considering taking part in this research. The person organising the research must explain the project to you before you agree to take part. If you have any questions arising from the Information Sheet or explanation already given to you, please ask the researcher before you decide whether to join in. You will be given a copy of this Consent Form to keep and refer to at any time.

- I understand that if I decide at any other time during the research that I no longer wish to participate in this project, I can notify the researchers involved and be withdrawn from it immediately.
- I consent to the processing of my personal information for the purposes of this research study. I understand that such information will be treated as strictly confidential and handled in accordance with the provisions of the Data Protection Act 1998.
- I agree to be contacted in the future by UCL researchers who would like to invite me to participate in follow-up studies.
- I agree that my non-personal research data may be used by others for future research. I am assured that the confidentiality of my personal data will be upheld through the removal of identifiers.
- I agree to donate a saliva sample for the purpose of analyzing my genes (DNA) to look for reasons why my sense of smell is the way it is.
- I know that the genetic analysis will be strictly limited and will not look for the genes of any known or suspected diseases.
- I understand the data from the genetic analysis will be irreversibly anonymised and the researchers will not be able to tell who it belongs to. There can be no information passed back about the findings on an individual basis, but the findings in general will be published.

Participant's statement

I [redacted]..(NAME) agree that the research project named above has been explained to me to my satisfaction and I agree to take part in the study. I have read both the notes written above and the Information Sheet about the project, and understand what the research study involves.

[redacted]..SIGNED.....28/07/15.....DATE

Researcher's Statement

I confirm that I have carefully explained the purpose of the study to the participant and outlined any reasonably foreseeable risks or benefits (where applicable).

[redacted].....SIGNED.....28/7/15.....DATE

*Figure.114: Musk family genetics sample consent form*

**Information Sheet:**

**Information sheet for: "**Investigating the genetics of human olfaction " **v 1.0:**

You will be given a copy of this information sheet.

Title of Project: Investigating the genetics of human olfaction

This study has been approved by the UCL Research Ethics Committee (Project ID Number): 1585-001

Name

Dr Simon Gane

Work Address

The Royal National Throat, Nose and Ear Hospital

330 Grays Inn Rd

London

WC1X 8DA

Contact Details

xNUMBER REDACTEDx

[redacted]

Who is doing this study?

These experiments are a collaboration between scientists at University College London and the Wellcome Genomics Centre Sanger institute in Cambridge UK.

The primary researcher is Dr Simon Gane, an ENT surgeon and form part of his PhD project in the Department of Physics at UCL.

Why are we doing this study?

The ability to smell certain compounds has been shown to depend on possessing the correct receptor for that compound. Some of these receptors have been identified, but many others are "orphans". We aim to use a series of odorants, all of which are food-grade or used in perfumery, to examine our volunteer's senses of smell. We will then use a limited sequence of their olfactory exome (all the genes which could be coding for their olfactory receptors) to see whether a variation in their sense of smell can be explained by a variation in their genes. If we cannot find a reason within those genes we can widen the search to include other genes too.

What do we hope to find?

We hope to find one or maybe more regions in the DNA which are genes for the receptor which we are looking for. These regions will be intact in the people who can smell the test odorants, but broken in those who can’t.

Why do we need families?

The total amount of DNA in any human cell is vast and we don’t understand enough of it to be able to tell what parts have what functions for all of it. Blood relatives share the same DNA code and so any differences in the sense of smell caused by genes will be fairly easy to see because the number of differences between relatives is small. In unrelated people there will be a lot of those differences and it would be more difficult to see which of those many differences changed their ability to smell.

What do I as a volunteer have to do?

To do this experiment we need three things: to show that you have a generally good sense of smell, to show whether or not you can smell a musk scent and to collect a saliva sample so we can look for the genes for the receptor.

The smelling test will consist of smelling 12 "sniffin sticks" and identifying their odors from a set of 4 choices for each stick. The sticks are like big marker pens and the test is easy for people with a normal sense of smell.

Identifying the test scent will just mean sniffing a bottle or some perfumery smelling strips and saying whether or not you can smell anything. We may ask you to pick that smell out of a group of three additional smells.

The saliva sample is collected by spitting into a bottle, it needs a good bit of spit, but that's all!

Who has approved these experiments?

The Research Ethics panel of the University College London has approved these experiments. The approval number is 1585/001

Are there any risks to me if I take part?

Some people can be sensitive to strong smells, please let your researcher know if that is the case for you.

Some people can be allergic to common perfumery ingredients, this is exceptionally rare, but if you know that you are allergic you should not take part in this study.

What happens to my information?

All the information we gather will be anonymised: the identifying parts - name, age and sex are all removed from the other data collected and stored separately by the researchers with a copy of your consent to be in the study. The only thing that anyone else will know is the results of the DNA and smell testing. You will not be identifiable from that information. This process is irreversible; we will not be able to give back any information about the results of a particular person (although obviously we can tell you how you did in the smelling tests).

As part of a commitment to open science the Sanger Institute releases all data collected within six months of the collection. Although the data is all anonymised we will still only release it in a "managed" format: on request to qualified individuals from recognized institutions.

What if I have any more questions?

Please ask the person who has given you this form, they will happily answer any questions.

Who do I contact if I have concerns about the study?

In the first place please contact Dr Gane by any of the methods given below.

If you don't feel you can talk to Dr Gane, please contact the site representative. Their contact details are given below.

Can I leave the study?

You can opt out at any time for any reason, or no reason at all. If you'd like us to retain what data we have collected or you'd like it all destroyed, tell us at the time of opting out. Unfortunately because the saliva samples are irreversibly anonymised, once they've been taken away, we won't be able to tell which is yours to destroy it, so they will be retained and analysed.

I'd like to take part, what do I do now?

Great! Thank you. Just sign the consent form and we can get started.

All data will be collected and stored in accordance with the Data Protection Act 1998.

Contact details:

Dr Simon Gane MBChB MRCS(Edin) DOHNS

[redacted]

[redacted]

[redacted]

[redacted]

xNUMBER REDACTEDx

University College London

Prof Valerie Lund

Professorial Unit

Royal National Throat, Nose and Ear Hospital

330 Grays Inn Rd

London

WC1X 8DA

xEMAIL ADDRESS REDACTEDx