Scent communication in the Eurasian otter (*Lutra lutra*) and potential applications for population monitoring.

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Doctor of Philosophy

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This thesis is dedicated to my brother Domonick; whose passion for nature inspired me, but whose short life prevented him from exploring it in the detail that life has allowed me to.

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Summary

Scent is thought to be the most important mode of communication for many mammalian species, including otters. Spraint surveys, used widely to monitor otter populations, describe distribution only. Discrimination between identity parameters through chemical analysis of scent marks has been conducted for many species but the feasibility of using this to enhance population monitoring has not been fully tested.

Scent presentation experiments were conducted on captive otters and provided some evidence that otters can determine the sex and reproductive status of the scent depositor. These experiments provide the first evidence of a potential role of both volatile and non-volatile compounds in otter communication, and revealed that otters learn where scent signals are likely to be presented.

A series of experiments were conducted to optimise methods for the chemical analysis of otter scent. A combination of scent sample collection from otter carcasses and captive otters enabled the description of the chemical nature of otter scent. Scent was associated with age, sex and reproductive status, but not with diet. Scent was associated with individual identity and in females, progesterone concentrations, suggesting a role in reproductive behaviours.

Spatial analysis at a national scale revealed differences in scent between genetically distinct subpopulations of otters. On a catchment scale there was no association between scent similarity and spatial proximity. Although scent differed significantly with individual identity, differences were not at sufficient resolution to allow discrimination between unknown samples collected in the wild. This thesis makes several major steps towards unravelling the complexity of otter scent communication and adds to the knowledge of otter biology and behaviour.

Contents

Tables

Figures

Abbreviations

AMDIS	Automated Mass Spectral Deconvolution and Identification System
DFA	Discriminant Function Analysis
DVB/CAR/PDMS	Divinylbenzene/Carboxen/ Polydimethylsiloxane
ELIZA	Enzyme-linked immunosorbent assay
GCMS	Gas Chromatography Mass Spectrometry
GLC	Gas Liquid Chromatography
MUPs	Major Urinary Proteins
NERC	Natural Environment Research Council
NIST	National Institute of Standards and Technology
PDMS	Polydimethylsiloxane
PCA	Principal Components Analysis
PerMANOVA	Permutational Multivariate Analysis of Variance
SPME	Solid Phase Microextraction
TIC	Total Ion Chromatogram
TLC	Thin layer chromatography
UPLC	Ultra performance liquid chromatography
VOCs	Volatile Organic Compounds
ZSL	Zoological Society London

Chapter 1
General Introduction

1.1. Chemical communication

Scent communication is the most important mode of communication for mammals (Albone, 1984; Brown and Macdonald, 1985; Wyatt, 2003). Odours may be in the form of excretory deposits such as faeces or urine, or secretions from skin glands. The function of scent marks is commonly associated with territoriality, although the theory that scent marks form an impenetrable barrier, repelling all competitors, is likely to be too simplistic. Gosling's (1982) Scent Matching Hypothesis states that when the odour of a conspecific matches the odour of scent marks in the vicinity, then that individual is likely to be the territory owner. This usually results in the invader retreating and therefore avoiding expensive aggressive behaviour. There are, however, many more chemical communication functions, and odours may communicate more than one of these. Other functions include: mate attraction, where odours signal reproductive status or dominance; navigation, where scent marks function as sign posts or trails; aggregation, to locate conspecifics or to recognise kin or group members; and defence, when odours are released to repel predators or to warn conspecifics of danger (all reviewed by Wyatt, 2003). Scent communication of relatedness could function in kin recognition and inbreeding avoidance or nepotism (Charpentier et al., 2008) and phenotype matching (Sun and Müller-Schwarze, 1998b).

There is behavioural evidence of scent discrimination in many mammalian species (e.g. badgers, *Meles meles*, Palphramand, 2007; giant panda, *Ailuropoda melanoleuca*, Swaisgood et al., 1999) and attempts have been made to characterise these scent marks for many years using ever more sophisticated chemical analysis techniques. In early studies, gas chromatography revealed differences related to sex, age and individuals from the tarsal scent of black-tailed deer (Müller-Schwarze, 1969). When reviewing individual recognition from mammalian scent gland secretions, Albone (1984) stated that although differences enabling individual recognition have been suggested, limitations such as differences not being stable over time reduced their value. He concluded that a general paucity of information exists on the chemical ecology of wild

mammalian species. Since Albone's (1984) review, knowledge of chemical communication in wild mammals has expanded considerably.

Intraspecific differences in chemical composition of scent marks have been found in a variety of mammalian orders: ungulates (Müller-Schwarze, 1969), rodents (Sun and Muller-Schwarze, 1998a, b), carnivores (Hagey and Macdonald, 2003; Burgener et al., 2009), primates (Scordato et al., 2007; Smith et al., 2001; Setchell et al., 2010), chiroptera (Safi and Kerth, 2003) and lagomorphs (Goodrich and Mykytowycz, 1972). The colour and viscosity of anal gland secretion can be used to identify beaver sex (Schulte et al., 1995). Colour and volume differences were found in badgers between seasons, sexes and some individual-specific parameters such as age and body condition (Buesching et al., 2002a). Gland secretions have been shown to code for group membership (Sun and Müller-Schwarze, 1998a; Buesching et al., 2002b), stable individual differences (Sun and Müller-Schwarze, 1998b), sex, age, reproductive status and season (Buesching et al., 2002b), and age of the scent mark (Buesching et al., 2002c). Ring-tailed lemur scent marks communicate genetic quality and relatedness (Charpentier et al., 2008), however, relatedness and chemical similarity may not be correlated in other species (Safi and Kerth, 2003).

The majority of the studies cited above have measured volatile organic compounds (VOCs), however, some authors state non-volatile compounds or compounds of low volatility may be the most useful chemicals in long-lasting or delayed communication (Yuan et al., 2004). Yuan et al. (2004) criticise other authors for only sampling volatile compounds. For non-volatiles to be used as part of scent communication, direct contact with the scent mark is necessary in order for them to be detected. If contact is not made, non-volatiles may function by binding to volatile compounds and modifying their release (Albone, 1984). Proteins in tiger urine are thought to perform this function (Burger et al., 2008).

Non-volatiles play a major role in mouse communication, particularly in individual recognition (Hurst et al., 2001; Brennan and Kendrick, 2006) and sex discrimination (Roberts et al., 2010). They are detected by the vomeronasal organ rather than the main

olfactory bulb (Brennan and Kendrick, 2006). When Major Urinary Proteins (MUPs) were first described in *Nature* in 2001 it was not clear if mice detected MUP type through volatile ligands or the MUPs themselves (Hurst et al., 2001). By preventing direct contact with scent marks Nevison et al. (2003) concluded that the ownership signal of mouse scent marks is non-volatile. An alternative hypothesis is that volatiles containing the ownership signal attract the mouse to the vicinity of the scent mark but that it is direct contact with a wet substance (urine) that acts as a tactile cue, and triggers the countermarking response. MUPs have also been described for cats (Miyazaki et al., 2006) but have not yet been described in other species (Janotová and Stopka, 2009). Long chain fatty acids of low volatility found in the anal glands of badgers and three mongoose species may perform a communication function (Davies et al., 1988; Decker et al., 1992). Despite very few examples from other species, it is common to extrapolate findings in rodent models to all mammalian species (e.g. Brennan and Kendrick, 2006; Luo et al., 2003). This may, however, be inappropriate, as there is a broad range of evidence of volatile compounds functioning in chemical communication; it is therefore likely that both volatiles and non-volatiles play a role in communication.

1.2. Animal population monitoring and whether odour can contribute

Measurements of the abundance and distribution of wild animal populations, as well as the population structure with regard to age and sex ratios, are recorded for a variety of research purposes. The ability to identify or measure the number of individuals, number of males and females etc. is a prerequisite to understanding the population dynamics of any species. Along with these research purposes, management of wild animal populations, for example, for conservation purposes or disease or pest control, are enhanced by accurate measures of population structure. Informed conservation and management decisions can only be made with accurate data on population abundance and distribution. Population estimates are fundamental to conservation success.

Estimation of wildlife population sizes can be problematic, particularly for cryptic, nocturnal terrestrial carnivores with large home ranges and many methods exist to estimate their distribution and abundance (Wilson and Delahay, 2001). Animal populations are surveyed or monitored in a variety of ways, largely depending on the

nature of the species and their habitats. For some wild animals it is possible to make direct visual observations and sexual dimorphism allows the number of males and females to be counted. For some species it is possible to observe the reproductive status, for example baboons, in which females develop conspicuous sexual swellings around the time of ovulation (Shaikh et al., 1982). For other species, differences between the sexes may not be so obvious, and observations are limited to estimates of abundance and distribution. There are, however, wild animals whose appearance, nocturnal activity or dense habitat, means that direct observation is not a viable method to monitor populations, unless the animals are trapped.

Traditionally, observation of indirect signs, (e.g. faeces, footprints, nests, feeding signs) have provided a means of assessing populations of less conspicuous species, however, the information that can be gained from these signs alone is usually limited to species distribution, habitat use and estimates of abundance. Technology has provided alternatives and enhancements to direct and indirect observation. Firstly, technology has been developed that enhances direct observations, for example binoculars, telescopes and more recently camera traps, which are frequently employed, particularly for species who fear humans or exist at low density in dense habitat (e.g. rain forest). Camera traps do have disadvantages, for example, they are expensive, may be stolen or destroyed and the information gained is limited to the characteristics which may be visually determined. Secondly, laboratory analysis of animal samples collected in the wild can provide a variety of detailed information. These samples may require animals to be trapped, for example blood or tissues, or the less invasive collection of faeces. The focus of analysis of animal samples for population surveys has primarily been on DNA and to a lesser extent, hormone analysis (Kohn and Wayne, 1997). Non-invasive genetic sampling to identify individuals has become a key technique in wild animal conservation and ecology (Kohn and Wayne, 1997; Taberlet and Luikart, 1999; Schwartz et al., 2007). Parameters that change within an individuals' lifetime, such as age, reproductive status and dominance, however, cannot be determined from DNA analysis.

The evidence that chemical differences in scent-marks relate to the identity of the animal that deposited the scent-mark, suggests that chemical analysis of scent-mark odours may provide additional information to the existing methods of wildlife surveys. Despite the now wide range of species whose chemical communication has been investigated, the possibility of applying this to monitoring has received little attention. A similar technique was, however, recently developed for using human scent as forensic evidence (Curran et al., 2010). Using VOCs to identify species has been suggested for canid scats (Burnham et al., 2008), and dogs trained to identify the scent of wildlife species' scats are used to assist conservation research (Wasser et al., 2004). Hagey and Macdonald (2003) suggest use of scent as a monitoring tool for giant pandas but this has apparently only been tested on one sample. Apart from this one study little mention has been made of using semiochemistry to monitor species in the wild. This study assesses the possibility of using chemical analysis to monitor populations of the Eurasian otter, *Lutra lutra*, (Linnaeus, 1758): a species of conservation concern and protected under the European Habitats Directive.

1.3. Study species: the Eurasian otter, *Luta lutra*

The Eurasian otter, *Lutra lutra*, is one of 13 otter species worldwide, all of which are members of the mustelid family. *Luta lutra* is the most widely distributed of all the otter species and occurs through Europe, Asia (now extinct in Japan) and parts of North Africa. The well documented decline of otters in the UK and Europe in the last century has been linked to bioaccumulation of persistent organic pollutants including organochlorine pesticides and polychlorinated biphenyls, although other factors such as food availability and habitat quality are also likely to have contributed (reviewed by Chanin, 2003). Levels of these pollutants in otters have since declined (Jefferies and Hanson, 2000, 2012; Roos et al., 2001) and UK otter populations have increased: the latest National Surveys found otter signs in 58.8% of sites in England (Crawford, 2010) and 89.9% of sites in Wales (Strachan, 2010), compared to 5.8% (Lenton et al., 1980) and 20% (Crawford et al., 1979), in the late 1970s, respectively.

Throughout much of their range *Lutra lutra* are nocturnal and elusive and this, along with low numbers in the last century, has made research into their social behaviour,

population structure or population size extremely difficult. Much information about otter behaviour and ecology is derived from anecdotal observations reported in books rather than peer-reviewed journals (e.g. Kruuk, 1995, 2006; Chanin, 1985; Mason and Macdonald, 1986; Laidler, 1982). Systematic observations were conducted in Shetland, but much of these data are not peer-reviewed (Kruuk, 2006). The bulk of research on otter behaviour therefore comes from observations of otters living on the coast, as they tend to be diurnal (feeding coincides with tides) and are therefore more readily observed than those living in freshwater habitats. Given, the difference in periods of activity, habitat and diet, there may be other differences in the behaviour between freshwater nocturnal otters and marine-feeding diurnal otters. Although a useful insight into otter behaviour, there is a limit to what these observations can tell us about the population structure and behaviour of otters living in freshwater habitats.

1.3.1. Otter communication

Anecdotal evidence and some empirical studies indicate that otters are mainly solitary and can range up to 40km (Erlinge, 1967, 1968; Green et al., 1984; Kruuk, 2006) with some individuals exceeding this (Durbin, 1998). More than one otter is usually only observed when males and females copulate, or when a mother is seen with cubs before they disperse at around one year of age (Kruuk, 2006). Encounters with conspecifics are therefore rare; the use of vocal or visual communication is limited. The more gregarious otter species (e.g. *Pteronura brasiliensis* and *Aonyx cinerea*) use auditory communication regularly (Duplaix, 1980; Davis, 1978). Eurasian otters do use auditory communication when in direct contact with each other, for example ‘wickering’ or ‘chittering’ during aggressive encounters or while cubs are play-fighting and mothers frequently whistle to cubs (Kruuk, 2006). The mainly solitary nature of Eurasian otters suggests that the principle mode of communication must be long-lasting and not dependent on the signaller and receiver being together.

Differentiation between scent marks is present in many species with similar ecology to otters, for example, pandas (Hagey and MacDonald, 2003, Yuan et al., 2004) and brown bears (Rosell et al., 2011) which are solitary, and beavers which are semi-aquatic (Sun and Müller-Schwarze, 1998a, b; Schulte et al., 1995; Rosell and Steifetten, 2004).

Behavioural and chemical evidence of discrimination between scent marks also exists for a variety of mustelid species. Anal gland secretions and faeces (that presumably contains anal gland secretion) are the most frequently studied media and age, sex and individual differences in mustelid odours are common (Table 1). The most studied species are ferrets and badgers, probably owing to their commercial use and impact. Despite otter species representing more than a fifth of the mustelid family, no chemical studies, and only two behavioural articles, have been published on the discrimination between otter scent marks. In a recent review of mammalian semiochemicals (Burger, 2005), mustelids were discussed, however the focus was on types of compounds present rather than communication function. The author did not report anything on *Lutra lutra* due to the lack of published research on scent communication in this species.

Otters largely communicate using spraint - a combination of faecal material and anal gland secretion (Kruuk, 2006). Even in group-living otter species, spraint is an important means of communication (Leuchtenberger and Guilherme Mourão, 2009). Spraint has many characteristics that suggest its function is scent communication. Firstly, spraints are small, for *Lutra lutra* typically less than 10cm long and 1cm across; if the purpose was simply elimination of food waste otters would produce fewer and much larger spraints. Secondly, spraint is deposited in prominent and easily found places for example elevated positions, at junctions of water ways or bridge points (Erlinge, 1967). Finally, otters show olfactory interest in spraint; otters can be observed approaching and sniffing spraint and latrine sites (Gorman et al., 1978, Kruuk, 2006, Trowbridge, 1983). Experiments placing spraint from captive individuals on top of wild spraints resulted in all being ‘countermarked’ within four days (Mason and Macdonald, 1986).

There is much discussion on the function of spraint but exactly what it communicates remains unclear. Otters exhibit intra-sexual territoriality (Erlinge, 1967) and, in Shetland, home ranges often overlap (Kruuk and Moorhouse, 1991). These social patterns suggest spraints are unlikely to function in strict territory marking. Kruuk

Table 1.1. Discrimination of scent marks: evidence from the mustelid family.

		Differences investigated and type of evidence found c=chemical, b=behavioural, n/c=chemical test but no evidence found, n/b = behavioural test but no evidence found				
Sub family	**Species**	**Age**	**Sex**	**Individual**	**Other**	**References**
7 mustelid species					Species (c)	Brinck et al., 1983
Lutrinae	North American river otter *Lontra Canadensis*		b		Species, social status (b) familiarity, relatedness (n/b)	Rostain et al., 2004
	Eurasian otter *Lutra lutra*				Freshness, own vs. others (b)	Rozhnov & Rogoschik, 1994
Mustelinae	Ferret *Mustela furo*		c	c		Zhang et al., 2005
			c		During breeding season only	Crump, 1980a
			b	n/b	Sex hormones increased investigation	Woodley and Baum, 2003
			b		Familiarity (b)	Berzins and Helder, 2008
			b			Cloe et al., 2004
	Steppe polecat *Mustela eversmanni*	c	c	c		Zhang et al., 2002a; Zhang et al., 2003
	Stoat *Mustela erminea*		c	c		Crump, 1980b
					Dominance (b)	Erlinge et al., 1982
			b			Spurr et al., 2004
	Siberian weasel *Mustela sibirica*	c	c	c		Zhang et al., 2002a; Zhang et al., 2003
	Mink *Mustela vison*	c	n/c	c	Only adult pattern is stable over time.	Brinck et al., 1978
			n/c			Zhang et al., 2002b
					Species (b)	Harrington et al., 2009
	Pine marten *Martes mates*		*n/b*		*Own vs. others (b), familiarity (n/b)*	*de Monte & Roeder, 1990*
Melinae	Badger *Meles meles*		*c*		*During some seasons only*	*Service et al., 2001*
		c	*c*	*c*	*Group membership, season, body condition and reproductive status, freshness (c)*	*Buesching et al., 2002b, c*
			n/c	n/c	Group membership (c)	Davies et al., 1988
					Group membership, greatest during breeding season, (b)	Palphramand, 2007

Italics = not anal gland or faeces. Articles detailing compounds only are not reported here. Behavioural differences are in response to scent, not differences in marking behaviour alone.

(1992) proposes that spraints signal the use of resources so that other otters will avoid a partly-depleted patch. Trowbridge (1983) found most spraints at the centre of activity of an otter's home range and through conditioning experiments with captive otters showed otters can distinguish between their own scent and that of others. There is recent evidence to support Kruuk's Resource Signalling Hypothesis from otters in the Mediterranean: sprainting intensity is positively correlated with food availability (Prenda and Granado-Lorencio, 1996; Remonti et al., 2011). Navigation and signalling of reproductive status for mate attraction are other suggested functions (Kruuk, 1992, 2006; Remonti et al., 2011).

Otters rub their cheeks on objects, for example stones, and scrape at specific sites (Kruuk, 1992); these behaviours have been used to suggest otters may possess other glands with a role in scent communication. It has been suggested, for example, that *Lutra lutra* has inter-digital glands (Kruuk, 2006) but histological examinations have never been conducted. Scratching at scent-marking sites may be better explained in terms of scratching to advertise scent mark location or to remove other scent marks (Gosling, 1982). Cheek-rubbing may serve to pick up rather than deposit scent marks, or to remove ectoparasites (which are often found around the muzzle, E.Chadwick pers. comm.). Proctodeal glands in addition to anal glands have been mentioned by Trowbridge (1983) and Mason and Macdonald (1986), both based on Stubbe (1970). It is possible that the original article was mistranslated as these glands have never been found during post mortems of over 1000 otter carcasses at Cardiff University (E. Chadwick pers. comm.).

Otters also deposit anal jelly. Gorman et al. (1978) seems to suggest this substance is a deposit from the anal sacs and is produced with a periodicity similar to the female oestrus cycle. This green or brown mucus is, however, known to originate in the gut (Trowbridge, 1983; Kruuk, 2006). Captive otters deposit anal jelly only after 12 hours of food deprivation when all other solid waste had been deposited (Trowbridge, 1983; Carss and Parkinson, 1996). Anal jelly may, therefore, be a vehicle for scent when there is no other solid waste available (Chanin, 1985, Trowbridge, 1983).

Urine may also play a role in otter scent communication. Otters have been observed to urinate on top of spraint piles (Kruuk, 2006) and reviews of chemical communication in mammals

cite many other species using urine in communication (Eisenberg and Kleiman, 1972; Albone, 1984; Brown and Macdonald, 1985; Wyatt, 2003; Müller-Schwarze, 2006). It is possible, however, that urine is not significant for all species' communication. In ferrets, which like otters are mustelids, males show no sex discrimination from urine but they do from anal scents (Cloe et al., 2004).

1.3.2. Anal glands

Like most carnivores *Lutra lutra* possess two anal sacs laying either side of the rectum with ducts opening just before the anus. All mustelids have anal glands with the exception of the sea otter *Enhydra lutris* (Albone, 1984). This may be due to the almost entirely aquatic nature of this species making scent communication impossible (Kruuk, 2006), especially for territory or resource marking. Otter anal sacs have apocrine and sebaceous secretory tissues (Gorman et al., 1978) and are not unlike those of mink (*Mustela vision*) (Albone, 1984). Sebaceous and apocrine glands are usually under hormonal control (Ebling, 1977).

Albone (1984) describes otter anal sac secretion as yellow, brown or green, gelatinous and being deposited with periodicity which reflects the oestrus cycle. Although his citation is unclear, Albone (1984) presumably is referring to Gorman et al. (1978) who describe anal jelly, and not anal sac secretion (see above). There is no other published description of anal gland secretions. There is some suggestion that the contents of the anal glands are deposited separately from faeces (Albone, 1984). Gorman et al. (1978) describes otters sniffing established latrine sites, urinating and/or defecating, and then ejecting part of the contents of the anal glands on top or to one side. Eurasian otters are also said to eject contents from their anal glands when extremely frightened or challenged, for example when trapped (Kruuk, 2006).

The first documented attempt to characterise the contents of the anal scent sacs of *Lutra lutra* was by Gorman et al. (1978). Thin layer chromatography (TLC), gas liquid chromatography (GLC) and gradient acrylamide gel electrophoresis were used to analyse secretions from two captive otters. Anal sac contents were mostly protein and acidic mucopolysaccharide from apocrine glands and lipid droplets from sebaceous glands. TLC showed standard monoglycerides, fatty acids, tryglycerides and sterol esters. GLC, but not TLC, showed

differences between individuals but they were not stable over time. Gorman et al.'s (1978) data were used by Brinck et al. (1983), who compared the compounds of the anal sac secretions of seven mustelid species. Brinck et al. (1983) found the *Mustela* species were very similar containing low molecular weight sulphur compounds. Badgers, otters and pine martens did not have these compounds and differed greatly from the *Mustela* species. TLC was employed by Fasano and Milone (1993) to examine the lipid composition of 11 spraints and some compounds were not present in spraints presumed to be collected 48 hours since deposition that were in newer samples.

Trowbridge (1983) dissolved spraints from three captive otters in diethyl ether and used GLC to look for similarities and differences between the otters in the chromatogram profiles (compounds were not identified). Although differences and similarities between individuals were found, conclusions were limited by the small sample size. The scent profiles from the same individual were relatively stable over a 26 day period; supporting the idea that spraint may be used for individual recognition.

More recently at Cardiff University, several undergraduate research projects have investigated the discriminatory power of volatiles from otter anal gland secretions. Meynell (1999), for example, suggested differences between individuals and sexes. With a larger sample size and using principle components analysis Bradshaw et al. (2001) showed that female samples clustered within male samples, severely limiting the discriminatory power of analysis. Bradshaw et al. (2001) also suggest that whilst pregnant or with cubs, females may want to disguise their scent perhaps to prevent infanticide. Due to gestation period and length of cub dependence there are perhaps only short periods of a female's life when she is receptive to a male and the female otter would undoubtedly want to advertise this. Both Meynell (1999) and Stevens (2000) used solvent extraction to sample otter anal gland contents although solvents dilute the sample and compounds may fall below detection limits. Sinkowski (2001) examined the anal gland secretions of seven mustelid species using solid phase microextraction (SPME) and gas chromatography mass spectrometry (GC-MS). SPME is relatively simple and easy to use and can detect compounds at very low concentrations. Sex and individual differences were found between 13 individual otters. Otters also showed the greatest variation of all the seven species. An unpublished PhD thesis from Hull University

(Davies, 2008) examined otter spraints from 17 captive otters and found sex and group differences, although analysis methods differed for some samples. Small samples sizes limit the conclusions that may be drawn from all of these studies.

1.3.3. Otter monitoring techniques

Because of their elusive nature, except in some coastal areas, direct observation is not a practical means of population monitoring for *Lutra lutra*. Standard techniques for monitoring otters in the wild involve looking for indirect signs (i.e. spraint, anal jelly, footprints and scrapes). Otter spraint has a distinctive smell (commonly described as hay or jasmine) that allows field surveyors to identify it. The scat of the American mink, another riparian mammal whose scat it could possibly be confused with, has a more unpleasant smell. DNA analysis has recently confirmed the ability of field surveyors to identify correctly the mink scat; none of 61 scat samples identified as mink were from otters (Harrington et al., 2010).

Between 1977 and 2011 five national surveys of otters have been carried out in England (Crawford, 2010) and Wales (Strachan, 2010), four in Scotland (Strachan, 2007) and two in Northern Ireland (Preston et al., 2006). The methods for these surveys were based on the findings of Erlinge (1967) regarding home range size where 600m lengths of river are selected at intervals of 5-8km and searched for otter signs. Results are expressed as the percentage of positive sites. These methods provide a broad indication of distribution, not to locate every otter; they allow changes in distribution to be mapped but reveal little about population structure.

The reliability and value of these spraint surveys have been widely discussed (Jefferies, 1966; Mason and Macdonald, 1987; Kruuk and Conroy, 1987; Wilson and Delahay, 2001). Attempts have been made to improve these techniques for example using spraint density as well as spraint site to form an ‘annual index of population’ (Mason and Macdonald, 1993) but, in general, this provided little information beyond that collected by simpler surveys (Chanin, 2003). Increasing the length of river surveyed to 1 km can increase the number of positive sites (Mason and Macdonald, 1987). To improve resolution when monitoring smaller areas (e.g. Special Areas of Conservation) the sampling interval can be reduced; Chanin (2003) suggests intervals of 1-3 km or even every bridge.

These standard techniques provide an indication of otter presence in an area but do not reliably provide information on numbers or population structure. The absence of spraints does not necessarily mean the absence of otters. The number of spraints found does not provide a direct index of the number of otters present (Jenkins and Burrows, 1980) nor is there a correlation between sprainting and the frequency of use of an area by otters (Kruuk et al., 1986). Although Mason and Macdonald (1987) refute this, stating that spraint surveys can be used as indicators of population status and habitat use, even they describe the use as only a broad indication. Logistical and ethical concerns as well as legislation prevent trapping and marking otters for research.

Faecal DNA analysis has been applied to identify otter individuals (e.g. Hung et al., 2004; Kalz et al., 2006; Prigioni et al., 2006) but this method has a low success rate. Dallas et al. (2003), for example, reported 20%, one of the lowest recorded for mammalian faeces and much lower than the 80-86% mean success from other carnivore species (Broquet et al., 2007). With such low success, population sizes could be hugely underestimated. There are measures that can improve the success but they are often impractical in the field, for example, collection of frozen samples (Hájková et al., 2009) or samples collected within 12 hours of deposition (Coxon et al., 1999). A higher success of DNA extraction can be gained from hair samples (up to 87%) but genotyping errors lower the overall success of this method (Anderson et al., 2006). The practicality of collecting hair samples has also yet to be tested outside captivity.

Other less commonly used methods also exist. Submerged infrared counters have been used to monitor otters (Garcia De Leaniz et al., 2006) but this relies on assumptions about body size that can only calculate a minimum census estimate, and the equipment needed is very expensive. Infrared video cameras can have some success on land (Guter et al., 2008) but a lack of recorded footage when spraints and footprints are found in camera view questions the reliability of some equipment. Cameras are also at risk of flooding and vandalism (Kean, 2007). Analysis of hormones has been used to provide sex data (Kalz et al., 2006) and snow-tracking can be useful in countries with high snow levels (Sulkava, 2007). Carcass collection

can provide some information on otter distribution and numbers (Dallas et al., 2003) but again the level of detail is limited (Madsen and Gaardmand, 2000).

National surveys have shown otters are increasing in Britain since their declines in the 1950s-60s (Crawford, 2010; Strachan, 2007, 2010; Preston et al., 2006). Considerable effort and resources have been put into conservation measures to increase numbers and distribution. If numbers of otters cannot be measured reliably the success of these different conservation efforts cannot be quantified. Clearly, there is a need for additional methods to enable detailed population monitoring and research (Hutchings and White, 2000), and to assess conservation efforts. Furthermore, understanding population structure and dynamics will enable better evaluation of threats to otters and the prediction of any future declines. Developing methods to assess otter abundance is one of five targets identified in the UK Biodiversity Action Plan for the otter; the investigation of scent analysis for this purpose is also included (JNCC, 2007). This could contribute to both conservation and research goals.

1.4. Potential applications of chemical knowledge of otter scent

Potential applications for information gained through chemical research into mammalian semiochemicals include use of attractants or repellents in order to manipulate wild behaviour for example for pest control (Müller-Schwarze, 1990; Shumake, 1977). As otter populations increase (Crawford, 2010; Strachan, 2010) they are increasingly coming into conflict with fisheries, resulting in economic cost to fishery managers and illegal culling of otters. There is increasing interest in non-lethal methods of control through olfactory-related deterrents. The importance of chemical communication studies to mammalian conservation was recently highlighted in a review but the potential is often not realised (Campbell-Palmer and Rosell, 2011) and is often limited by the absence of data on the chemical composition of scent marks. Rostain et al. (2004) urge the need for investigations of otter olfactory signals in developing more productive methods to determine the status of otter populations; understanding the function could help to correct population estimates based on spraint surveys.

An accurate assessment of population structure based on scent marks relies on all types of individuals scent marking at similar rates. Bias would arise if not all groups within a

population deposit spraint in equally conspicuous places. If the Scent Matching Hypothesis (Gosling, 1982) is true for otters, where scent is used as a means for intruders to identify territory residents and therefore avoid agonistic encounters, then it is unlikely that non-resident adults and juveniles would spraint as frequently as residents. There is some suggestion that female otters spraint in the water when they have cubs, to prevent detection (Kruuk, 2006, Ostman et al., 1985). If these groups were missed then population size would be underestimated and population structure would be adult and possibly male-biased. Individual heterogeneity affecting the probability of an individual being sampled has been identified as a pitfall in population estimates but adjustments to sampling protocols can reduce this (Ebert et al., 2010). There is evidence, however, that this may not be necessary for otters. Male, female and juvenile otters have been observed sprainting at similar rates (Kruuk, 2006). DNA analysis of wild collected spraints has identified female otters (Dallas et al., 2003) and hormone analysis has identified juvenile and pregnant female otters (Kalz et al., 2006). Marking behaviour has been seen in cubs as young as eight months (Erlinge, 1968). These findings support the use of spraint for population monitoring.

Identification of individual otters through chemical analysis of spraints would allow better monitoring. If chemical analysis of otter spraints can discriminate individuals, this would overcome the potential problem of over-counting individuals that spraint more frequently. Individual identification could be used to map home ranges and movements of animals; this has previously only been possible with radio tracking. Information about the owners of spraints (for example their age or sex) could be combined with habitat information to provide clearer information on habitat use and requirements, and therefore inform conservation.

Non-volatile compounds have been investigated previously with the view to distinguish between individuals and home ranges of wild otters. Although differences between individuals were stable over time, inevitable contamination in the field limits the use of gradient acrylamide gel electrophoresis (Gorman et al., 1978). Unsuccessful attempts were made to overcome contamination issues by using two dimensional immuno-electrophores (Jenkins et al., 1981). As yet analysis of VOCs for population monitoring of otters has not been attempted. The anecdotal accounts of otters sniffing spraints make no mention of otters making direct contact with spraints (Gorman et al., 1978; Trowbridge, 1983; Kruuk, 2006)

which would be necessary for the detection of non-volatiles. It therefore seems likely that at least part of communication is achieved through volatile compounds and analysis of VOCs with the view to enhancing population monitoring is worthy of investigation.

1.5. Aims

This thesis aims to optimise methods for sampling VOCs from otter scent, to describe this scent and explore differences in scent according to otter identity and biology. It also aims to infer what information is communicated between otters. In doing this, the thesis represents the first steps towards the ultimate aim to develop a monitoring technique that can identify otters through chemical analysis of spraint. Anal gland secretions and spraints are studied: urine marks would be unsuitable because they are difficult to identify or collect in the field, and are easily washed away. This also suggests that urine would not be as useful as spraints for long-term communication. Given the differences found in the scent marks of various species, including otters, it is expected that discrimination of otter sexes, age groups and individuals may be possible and that these differences may be used to monitor populations in the wild.

1.6. Thesis structure

Chapter Two reports a test of the scent discrimination ability of otters in captivity. These behavioural tests complement the chemical analysis in other chapters, by testing the relevance of scent to otters.

Chapter Three describes several sequential experiments optimising the use of SPME to sample otter scent. The impact of storage conditions are explored and explores (because many samples used in subsequent experiments originated from otter carcasses) how scent changed in a decaying carcass. The impact of otter diet on scent is also tested. The Chapter concludes with recommendations for developing methods and the methods used in subsequent chapters.

Chapter Four describes categorical differences in the scent of otter anal gland secretions according to age, sex and female reproductive status. This Chapter is published as Kean, E.F.,

Muller, C.T. and Chadwick, E.A. 2011. Otter scent signals age, sex and reproductive status. *Chemical Senses* 36(6), 555-564.

Chapter Five details an assessment of individual differences in otter scent; exploring the existence of an individual scent fingerprint; how this varies over time and (in females) with progesterone.

Chapter Six examines spatial variation in scent on a national England and Wales scale and relates this to genetic subpopulations. Smaller scale spatial variation is explored using field collected spraint.

Chapter Seven draws these findings together in a general conclusion and suggestions are given for future research directions.

Chapter 2

Responses of Eurasian otters to intraspecific scent marks

2.1. Introduction

Relating the data from chemical analysis of scent marks to known information about the scent donor allows suggestions of what information animals communicate through scent. The chemical differences detected by laboratory equipment, however, may not be those detected by the animals. Behavioural evidence of animals' ability to discriminate between different scents exists for a variety of mammalian species (e.g. badgers, Palphramand and White, 2007; giant pandas, Swaisgood et al., 1999). A combination of chemical analysis with behavioural experiments or observations (e.g. banded mongooses, Jordan et al., 2010; spotted hyaena, Burgener et al., 2009) provides a much more powerful investigation of a species' scent communication. Chemical analysis of scent marks allows differences in scents to be characterised and quantified, while behavioural experiments can reveal which of these differences are relevant to that species or individual. Combining the two disciplines enhances scientific investigation.

Behavioural experiments, or bioassays, have provided evidence of the ability to distinguish female reproductive status from scent in several mammalian species. Male African elephants, for example, respond more to female urine around the time of ovulation (periovulatory) than urine from the non-oestrous phase (Bagley et al., 2006), as do male giant pandas (Swaisgood et al., 2002). There is also evidence in mammals of females' responses to scent differing with their own oestrous stage (e.g. giant pandas, White et al., 2003). Rostain et al. (2004) demonstrated the scent discrimination ability of male North American river otters by presenting different scents to otters and measuring the amount of time spent investigating. Males of this species showed evidence of species, sex and social status discrimination. Rostain et al. (2004) propose further research is needed to test the role of spraint in otter sexual communication. The relationship between otter chemical signals and reproductive hormone concentrations is not fully understood.

Differences in the scent communication behaviour of male and female otters have been observed in captive otters: in responses to deposits and patterns of depositing. An increase in

sprainting activity has been observed when new male otters are placed into enclosures; total spraints in enclosures of groups of otters is lowest when cubs are born and highest when cubs are 5-6 months old (Prigioni et al., 1995). The authors suggested that this means there is a relationship between sprainting activity and the breeding cycle. Cubs start sprainting independently at 6 months old (Polotti et al., 1995) and male cubs spraint more than females; at 5-6 months females were depositing faeces randomly, while male cubs sprainted in a heap and refreshed it within 5 minutes of it being removed (Green, 2000).

Being able to determine how recently a scent mark has been deposited could allow an animal to assess how recently a conspecfic has visited that area and therefore how relevant the information is. This may be particularly relevant for frequently changing information such as reproductive status. Unsuccessful attempts were made by Rozhnov and Rogoschik (1994) to demonstrate the ability of *Lutra lutra* to distinguish fresh spraints from 7 day old spraints; their failure could have been due to aspects of their experimental design. Rozhnov and Rogoschick (1994) presented two spraints originating from the test animal and so any difference in freshness might not be relevant to that individual. An individual may use memory to know how recently it has visited a site but would require external information, such as scent, to determine how recently a conspecific had been in the area. Testing for recognition of freshness of another individual's spraint may be more relevant. Additionally, Rozhnov and Rogoschik (1994) used two separate spraints in these tests so, had different responses been seen, these could be due to dietary or reproductive changes rather than just freshness. Further tests did show that otters were able to distinguish between their own and other spraints that were up to 30 days old, but not as much as 60 days (Rozhnov and Rogoschik, 1994). A more appropriate method might be that used by Buesching et al. (2002c) to describe changes in the chemical composition of badger scent marks over time by dividing samples into six subsamples and freezing them after different periods of time.

An attempt has been made to test *Lutra lutra's* ability to discriminate between individuals by training an otter to display conditioned responses to certain spraints (Trowbridge, 1983). The test male otter distinguished between his own and another otter's spraint, spraint of known and unknown otters and two different unknown individuals, however, conclusions from this are obviously limited by sample size. Some tests have been conducted on *Lutra lutra* to

investigate it's response to unfamiliar spraints (Durbin, 1989; Mason and Macdonald, 1986) but again conclusions are limited by small and unrepresentative sample sizes (n=2 females). Furthermore, there have been no studies on *Lutra lutra*'s responses to the spraints of different sexes or different reproductive stage.

To investigate if sex, reproductive stage or freshness of spraint is communicated between Eurasian otters, captive otters were presented with different pairs of scents. The hypothesis was that otters would investigate one spraint more; the null hypothesis was that there would be no difference in duration of time spent investigating the different scents. To control for any differences in scent other than freshness, the same spraint was divided into two; half was allowed to age, improving on the experimental design of Rozhnov and Rogoschik (1994). The amount of interest in the control poles over time will be examined to investigate if otters change their amount of investigation as they gain more experience of where to expect scents to be located.

2.2. Methods

2.2.1 Study animals

All otters (n=12) were housed at New Forest Wildlife Park, Hampshire, UK in outdoor enclosures with indoor dens. Otters were housed in pairs; five male-female pairs (one pair had only just been introduced), and one female-female pair. All of these otters were rescued from the wild. Three pairs were in enclosures on show to the public and three were off-show. These off-show otters will be released back into the wild when old enough and suitable release sites are identified. The on-show otters are not suitable for release into the wild and will remain in captivity. The age of the otters ranged from eight months to 13 years (Table 2.1).

2.2.2 Scent samples

Three pairs of scents were presented to the otters, testing response to progesterone level, age of spraint and sex. For the progesterone level experiment, spraints were collected over a period of three months from a single female otter housed at the Centre de Réintroduction des Cigognes et des Loutres, France and progesterone metabolite analysis was conducted on samples every other day (Chapter 5). Samples of high and low progesterone were selected.

Table 2.1. Details of otters exposed to scents in order to investigate responses to scents.

Enclosure	Otter	Age	Sex
1 On-show	Topaz	3 years	Female
	Trooper	3 years	Male
2 On-show	Sparky	11 years	Male
	Doris	8 years	Female
3 On-show	Damsel	13 years	Female
	Lancelot	13 years	Male
4 Off-show	Grace	19 months	Female
(recently introduced)	Sirius	10 months	Male
5 Off-show	Caliban	8 months	Female
	Terra	8 months	Female
6 Off-show	Sixpence	21 months	Female
	Arven	19 months	Male

The spraint samples for the sex experiment were collected from a male and female otter at the New Forest Wildlife Park (these donor otters were not included in this experiment). Samples for the spraint freshness experiment were collected from otters at the New Forest. Only spraints that appeared fresh (i.e. still wet and often warm) were collected. To standardise conditions, and control for variations in internal state (e.g. hormonal or diet), samples were split in half. Half was returned to the freezer and half was left exposed indoors for 4 days before being returned to the freezer. Spraints collected for the progesterone level and freshness experiments were stored at -20^{o}C in sterile 7ml plastic bijou tubes for 12 months before the scent presentation experiments. Samples were removed from the freezer approximately 24 hours before the start of the experiments and were kept in a cool bag with ice between presentations. Spraints for the sex experiments were collected 24 hours before the start of the experiments and also kept on ice.

2.2.3 Scent presentation

Small amounts of the spraint samples (approximately 1cm^3) were placed on white duct tape (50mm wide) around the end of wooden poles (120 cm in length) that were pushed through the enclosure fences. This tape was removed and refreshed between experiments. The poles were only used in one enclosure to prevent transfer of smells between enclosures. Gloves were worn when handling equipment to prevent human scent being transferred. Three poles were presented in each experiment; two with scents and one control (with tape only). The

poles were placed 120cm apart (Figure 2.1). Experiments were conducted blind so that the person recording the data didn't know the contents. Each experiment was conducted only once on each pair of otters. Repeatedly using the same subjects in play back experiment is considered pseudo-replication (Hurlbert, 1984) unless statistics are based on the number of animals tested rather than the number of responses measured (Kroodsma et al., 2001). The resources and time available for this experiment did not all repeated presentations. The order in which the experiments were conducted was randomised to control for effects of prior experience. The placement of the two test poles and one control pole were randomised to control for position effects (i.e. the control pole was not always be in the same position).Taped poles were placed in the enclosures without any scent samples 24 hours before the experiments began allowing the otters to become accustomed to their presence and ensure the reactions recorded were to the scents and not the poles. Some otters were nocturnal and some more diurnal, individually specific periods of activity were identified in consultation with the Wildlife Park keepers and scent presentation experiments concentrated during these times.

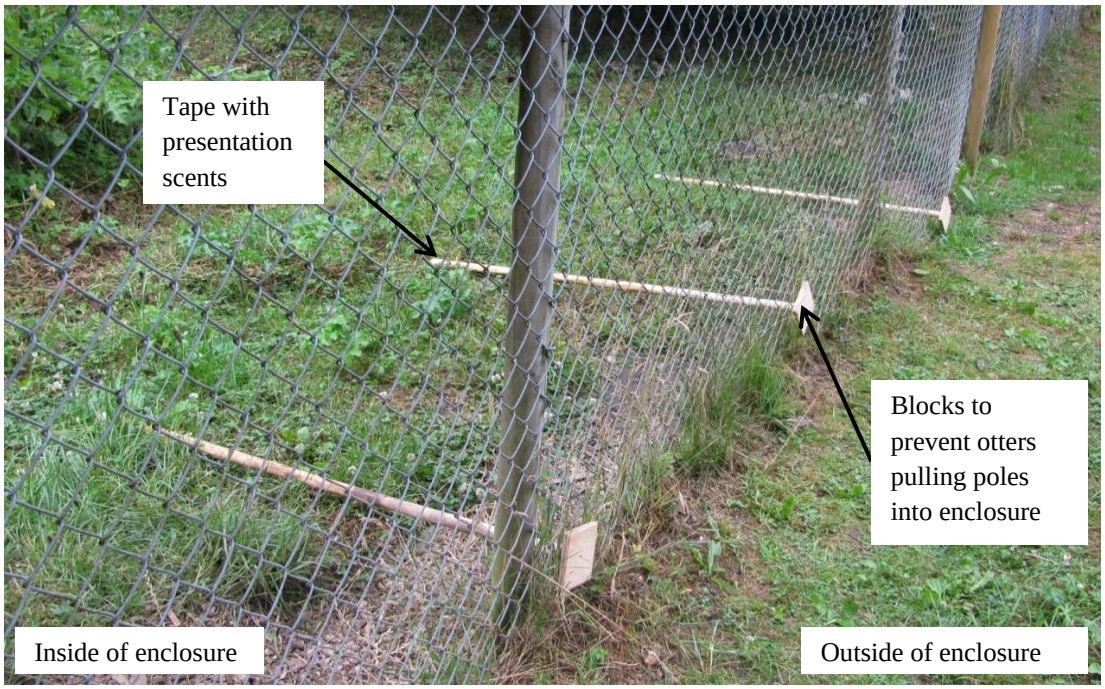


Figure 2.1. Scent playback experiment equipment showing poles used to present scents pushed through boundary fence of otter enclosure.

2.2.4 Data collection and analysis

Video cameras were used to record the behaviour of the otters. Cameras started recording as the scent presentation poles were pushed into the enclosure. A Sony Handycam (DCR-HC 36) was used to record the scent presentations on the more diurnal otters for a total of 45 minutes from when the scents were placed in the enclosure. For nocturnal otters two models of remote sensor infrared cameras (Stealthcam) were placed along with scents in the enclosure for approximately 18 hours, including overnight. The duration (in seconds) and the number of bouts of investigatory behaviours within 50 cm of, and directed towards, the scent poles were recorded. Investigatory behaviour was defined as the otter sniffing the air, ground or pole. Sniffing is characterised by head being directed towards stimulus with slight bobbing of head or flaring of nostrils. The occurrence (count) of countermarking (the depositing of spraint, urine or anal jelly within the 50cm) was also recorded. Data were analysed in R version 2.13.1 (R Development Core Team) using Friedman tests with post-hoc tests with correction for multiple comparisons (Wilcoxen-Nemenyi-McDonald-Thompson). A non-parametric test was chosen due to the small sample sizes. Friedman is a repeated measures test, which tests for differences between mean ranks, so testing the consistency of the trend, rather than the variability between subjects. For this reason parallel coordinate plots are presented rather than means with error bars.

2.3. Results

It was not possible to distinguish between the two females housed together, these were also the youngest otters tested, so the data collected from these two otters were not included in further analysis. Some of the otters showed no response to the scents presented (1/6 otters for sex experiment, 2/10 for progesterone experiment, 4/8 for the aged spraint experiment). It was not possible to video record for longer than 30 seconds on the Stealthcams used so duration data were not always reliable (2/10 otters for progesterone experiment). Otters typically sniffed the air whilst walking towards the pole, then made direct contact with the pole with their nose (Figure 2.2). Direct contact was characterised by repeatedly dabbing the nose on the pole or ground surrounding the pole.

Figure 2.2. Otters making direct contact with scent.

2.3.1 Sex

Five otters responded to the sex experiment; two females and three males. There was a significant overall effect of test scent (Figure 2.3; maxT=2.53, p=0.03). Four of the five otters spent most of their investigation time on the female scent, the other otter only spent 1 second longer on male scent than female scent. Post-hoc tests showed that otters spent significantly more time investigating female scent than controls (p= 0.03). There was no significant difference in investigation time between male scent and control (p=0.42) or female scent (p=0.42). There was a significant overall effect of test scent on number of bouts (Figure 2.3; maxT=2.57, p=0.03). Post-hoc tests showed there were significantly fewer bouts of investigation on control scent than both female (p=0.03) and male (p=0.03), but there was no significant difference in number of bouts between male and female spraints (p=1.00).

2.3.2. Progesterone

Six otters responded to the progesterone experiment; three males and three females. There was a significant overall effect of test scent (Figure 2.4; maxT=3.46, p=0.002). Post-hoc tests showed that otters spent significantly more time investigating low progesterone scent than control (p= 0.002). There was no significant difference in investigation time between high progesterone scent and control (p=0.19) or low progesterone scent (p=0.19). There was no significant difference in the number of investigation bouts (Figure 2.4; maxT=1.51, p=0.29).

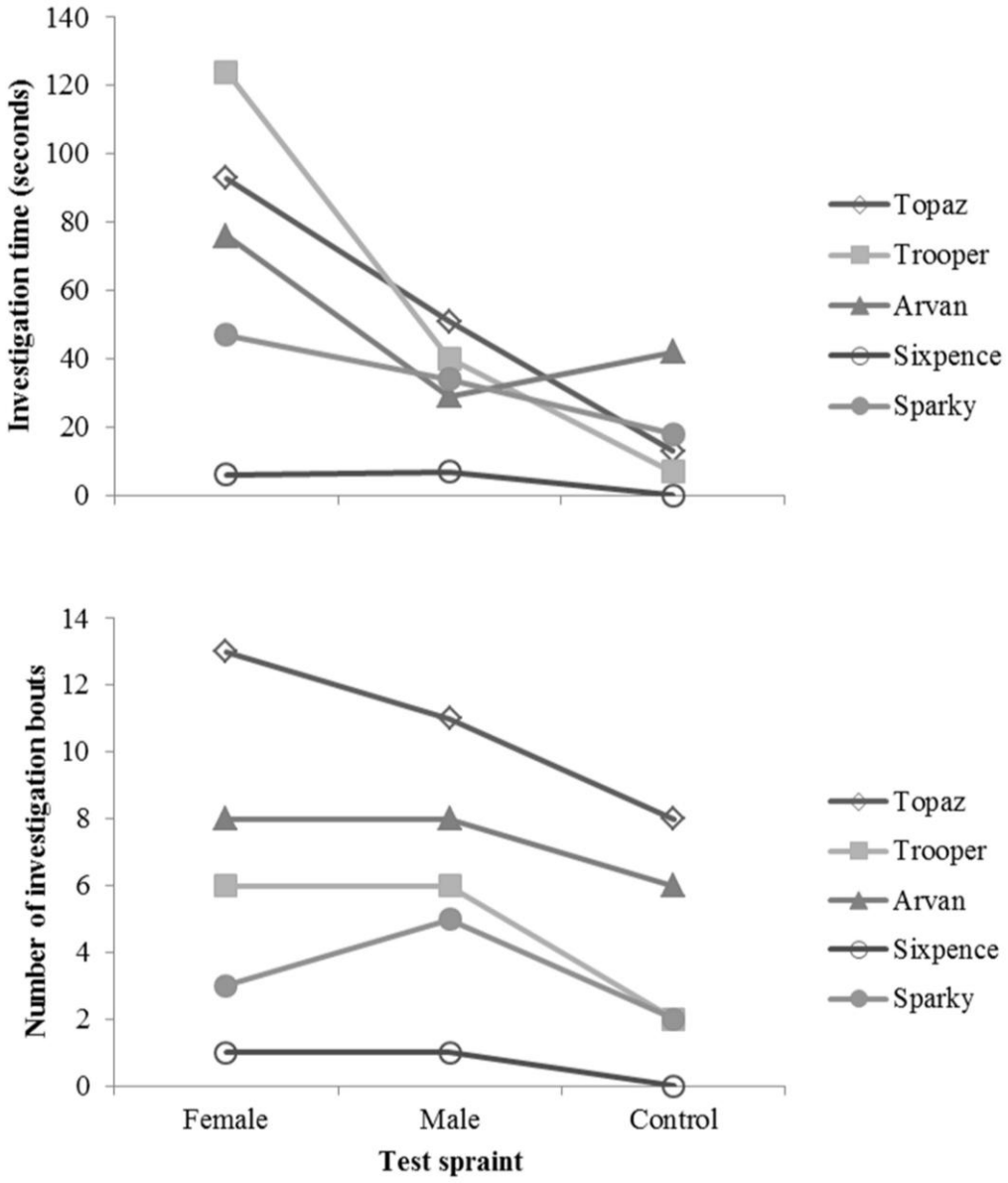


Figure 2.3. Parallel coordinates plots of the amount of time otters spent investigating and the number of bouts of investigation of male, female and control scents. Each line represents an individual otter. Male test otters are indicated by filled symbols, females are indicated by open symbols.

2.3.3. Freshness

Four otters responded to the freshness experiment; two males and two females. There was no overall effect of test scent on investigation time (Figure 2.5; maxT=2.12, p=0.086), however, this was close to statistical significance. There was no significant difference in the number of investigation bouts (Figure 2.5; maxT=0, p=1.00).

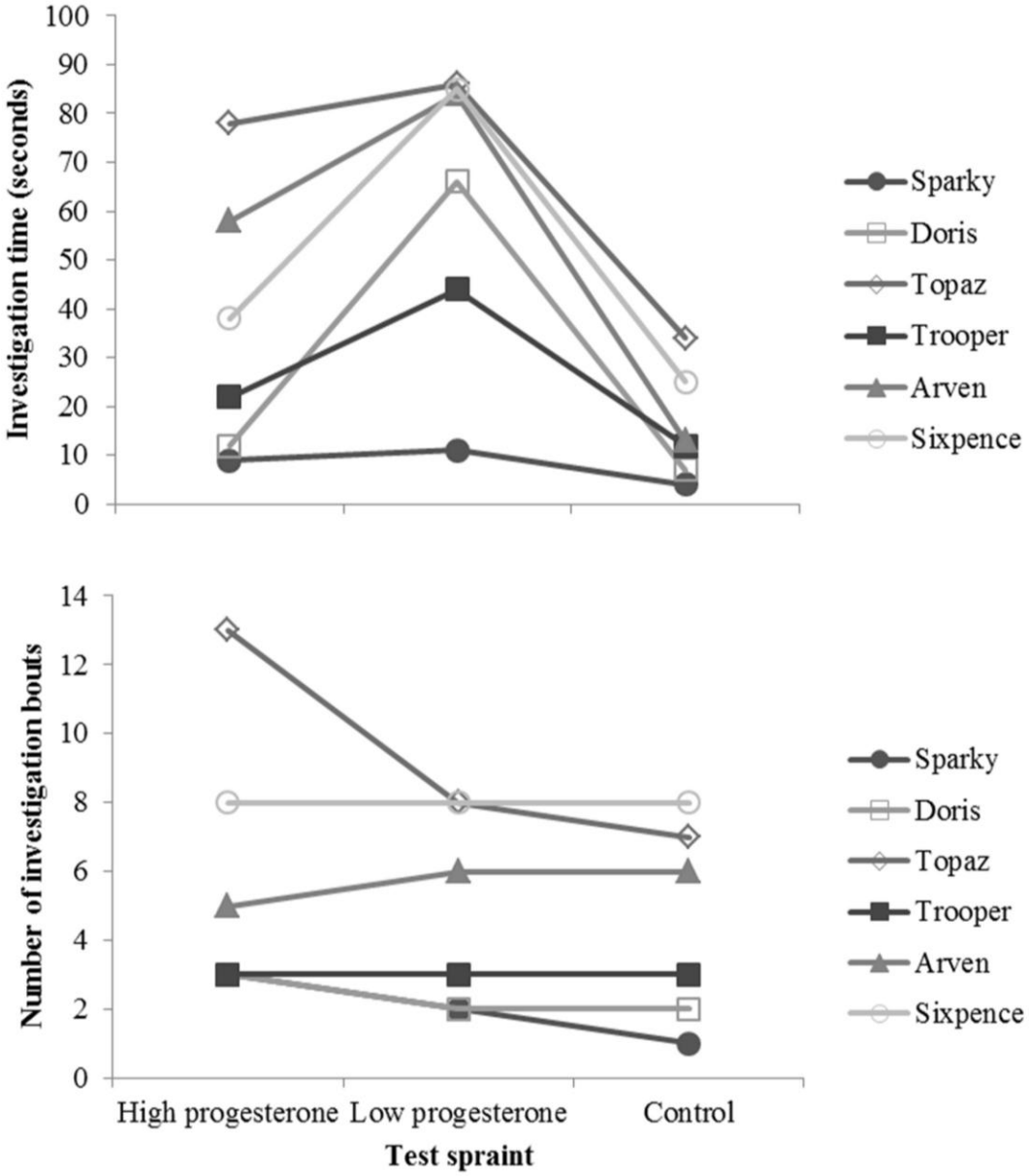


Figure 2.4. Parallel coordinates plots of the amount of time otters spent investigating and the number of bouts of investigation of high progesterone, low progesterone and control scents. Each line represents an individual otter. Male test otters are indicated by filled symbols, females are indicated by open symbols.

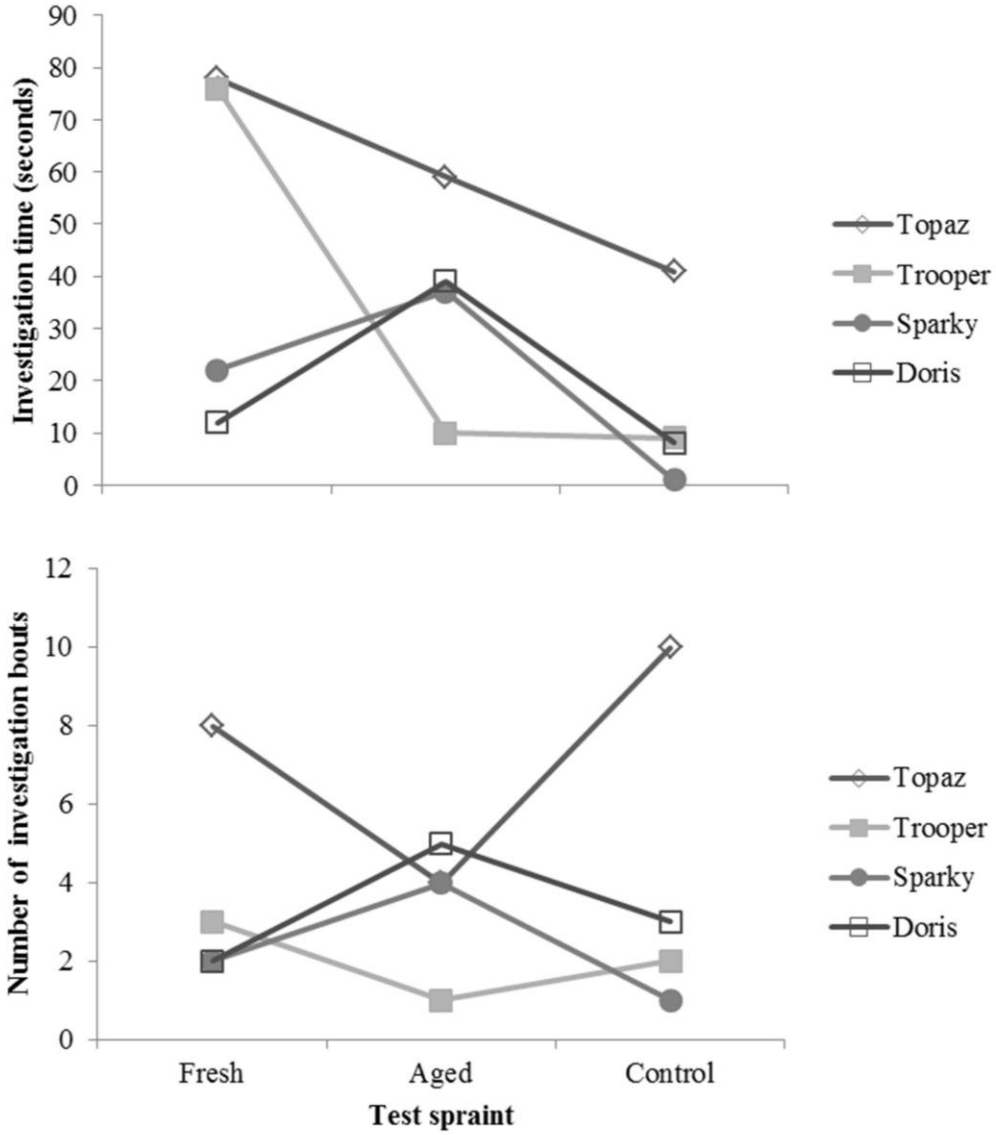


Figure 2.5. Parallel coordinates plots of the amount of time otters spent investigating and the number of bouts of investigation of fresh, aged by 4 days and control scents. Each line represents an individual otter. Male test otters are indicated by filled symbols, females are indicated by open symbols.

2.3.4. Experience of experiments

There was some evidence that with more experience of the experimental set-up, otters showed more interest in the control pole. Four out of six otters increased their investigation of the control pole over time (Figure 2.6), regardless of location. One otter decreased slightly (Doris); this is an old female who did not respond to any of the scents in the third experiment. One other otter (Arven) investigated the control pole in the second experiment for 42 seconds and only 13 seconds in the third experiment.

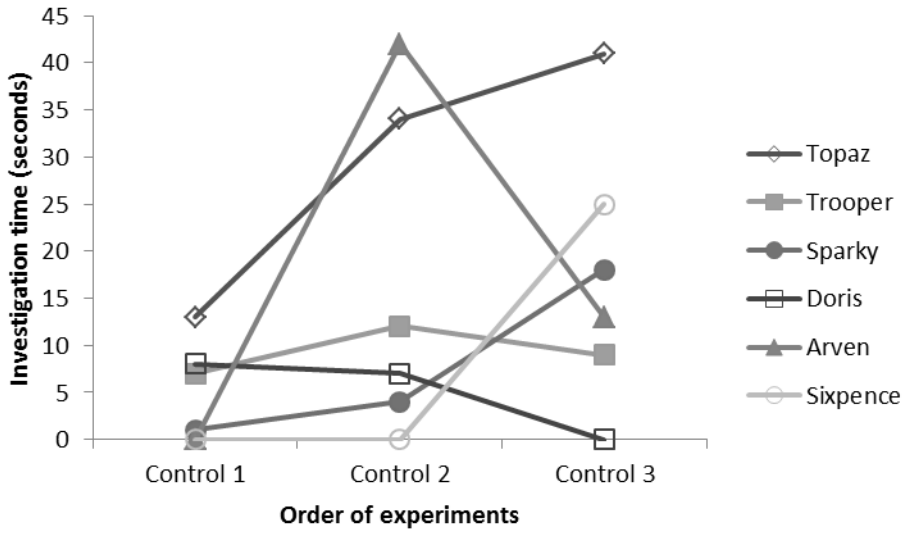


Figure 2.6. Parallel coordinates plot showing the time otters spent investigating control scent (no spraint) in three experiments, each approximately 24 hours apart. Male test otters are indicated by filled symbols, females are indicated by open symbols.

2.3.5. Countermarking

Nine instances of countermarking were recorded over 22 scent presentation trials (one trial is a presentation of two scents plus control to one otter). Of these, two were next to a fresh spraint (by one otter), two were next to a male spraint (by one female and one male otter) and four were next to high progesterone spraints (two by one male otter and one each by two female otters). In a preliminary experiment (data not presented here) with a total of 18 scent presentation trials, where direct contact with the scent was prevented, countermarking was only observed once.

A further eight instances by two otters were recorded, however these were discounted because although they were within 50cm of the pole, they were on top of an existing spraint pile, already used by these otters.

2.4. Discussion

These experiments suggest Eurasian otters possess the ability to distinguish between the sex and reproductive status of the spraint depositor and to learn the location of spraints. They also provide the first evidence of a potential role of both volatile and non-volatile compounds in otter scent communication. Like many other studies on the scent discrimination of wild animals, however, the conclusions are limited by small sample size (n=4-6). There is no

Friedman's test specific power analysis, to estimate either the reliability of the results here, or the required sample size to gain sufficient statistical power. The results presented should, therefore, be viewed with caution. Some try to overcome small sample sizes by repeating experiments on the same individuals (Rozhnov and Rogoschik, 1994; Rozhnov and Rozhnov, 2003; de Monte and Roeder, 1990), however, as these replicates are not statistically independent, this is pseudo-replication. To avoid pseudo-replication, in this experiment, each scent presentation was conducted only once on each otter, even though this meant sample sizes were small. Herr et al. (2006) used the same method to avoid pseudo-replication in conducting scent play-back experiments on free living beavers. In the current study, one result was close to significant; the overall effect of test scent on investigation time in the freshness experiment ($p=0.086$). The small sample sizes meant very conservative non-parametric tests had to be employed; with larger sample sizes, less conservative and more powerful parametric tests (e.g. 2 way repeated measures analysis of variance) this near significant result could be clarified. The initial sample size ($n=12$) was reduced because some otters did not respond to some of the experimental treatments. This is typical of otters in captivity, who are not very frequently active, typically confining activity periods to feeding times (Laengle and Jorga, 2003). In the experiments where otters did not respond, they were inactive and resting in their dens so did not encounter the scents. Although this pattern of behaviour makes experimentation difficult, it is not atypical compared to otters in the wild, who tend to conserve energy by often resting in holts and couches (Kruuk, 2006).

For the sex and progesterone experiments only post-hoc text was significant; one of the test scents was investigated for significantly longer than the control. As there was no significant difference in investigation time between the two test scents in these experiments, trends in investigation time are not strong and are difficult to interpret. Otters spent significantly more time investigating female spraints than control but not significantly more than male spraints, although 4 out of 5 otters tested spent more time investigating female rather than male scent. The number of bouts of investigation did not differ between male and female spraints. In contrast to the male Eurasian otters in this study, male North American river otters (*Lontra canadensis*) spend more time investigating other male rather than female scent (Rostain et al., 2004). The similar olfactory interest in female spraint by both males and females (4 out of 5 otters spent more time investigating female than male spraints) suggest that male otters use spraints in intersexual communication, for example, to locate a mate, and female otters use spraints in intrasexual communication, for example interindividual spacing. This fits with

current understanding of the spatial organisation of otters in the wild where females have exclusive ranges and males overlap several female ranges (Erlinge, 1967, 1968; Kruuk, 2006).

There is evidence from many other species that mammals can determine oestrous stage from scent marks (Bagley et al., 2006; Swaisgood et al., 2002; Clarke et al., 2009; del Barco-Trillo et al., 2009). Unfortunately, the hormone metabolite analysis conducted in this thesis (Chapter 5) did not allow the determination of oestrous stage as no oestrogen metabolites were detected (both progesterone and oestrogen levels are necessary to confirm oestrous stage). Progesterone concentrations were measured and the results of these behavioural tests indicate that otters might be able to detect the differences between high and low progesterone concentrations. Otters spent significantly more time investigating the low progesterone spraints than the control, but not significantly more than the high progesterone. Although not conclusive, the results indicate this would be worthy of further investigation.

The otters showed no difference in investigation time between fresh and 4-day aged spraints. Additionally, there was no difference between investigation time between either of these and the control. This may suggest that after 4 days, otters can gain the same information from spraint as if it were fresh. The results confirm previous findings of no difference in response between fresh and old spraint, although those authors used different spraints, rather than splitting the same spraints (Rozhnov and Rogoschik, 1994). It would be interesting to investigate this further to see how long lack of difference is maintained, and if it is true for all spraint types, for example from males and females, familiar and unfamiliar.

Many of the test scents were not investigated for significantly longer than the control. This does not necessarily mean that those test scents were not of interest to otters; rather, it means that the control was of equal interest. From the observed increase in interest in the control with more experience of the experimental setup, the otters appear to expect a scent to be presented on each pole; they learn where to expect spraints to be. In the wild, otters re-use the same sites for depositing spraints. A similar pattern of learning where to expect scents to be presented has been observed in mice (J. Hurst pers. com.).

The results presented here and that of many similar experiments assume that time spent investigating scent is a good measure of discrimination. Rostain et al. (2004) hypothesised,

when referring to the scent of oestrous, that some scents may be so distinct that only a short investigation is needed to detect the information. Length of time investigating scent is simply a measure of information gathering, not what that information is. The idea of distinct scents requiring short investigation presented by Rostain et al. (2004) could be true of other scents. Measuring the frequency of over-marking is an alternative method that may give a clearer indication of scent function (Jordan et al., 2010). In the present study, countermarking was observed only on the test scents that were investigated the least; the male and high progesterone spraints. This again shows that time spent investigating may not be a useful measure of the otters' ability to discriminate between scents, or a preferential interest. An additional measure would be to record the animal's behaviour after investigating the scent marks; for example, does investigating certain scent marks result in increased exploration of their enclosure. In these experiments some otters, following investigation of presented spraints, were observed to spraint on existing spraint piles that were outside the 50cm range of recorded behaviour. The range of the camera did not allow this to be recorded for all otters in this experiment.

Some species over-mark directly on top of the scent marks of conspecifics, others counter-mark next to the scent marks of conspecifics. The reason for using one strategy over the other is not clear. Johnston et al. (1994) described three different types of over-marking; direct over-marking resulting in a blended group scent, counter marking adjacent to other scents to maintain a distinct individual signature and thirdly over-marking to mask the smell of others. It could be the social organisation of a species that determines which type of counter-marking is used; indeed the species referred to by Johnston et al. (1994) for countermarking to maintain distinct individual signatures are mostly solitary. The otters did not counter-mark by depositing spraint or urine directly on top of the presented scents, rather it was deposited adjacent to the test scents, at a distance of approximately 30cm. Mice, a mammal species whose countermarking has been more extensively studies, do not over-mark directly on top of another mouse's scent mark (Hurst, 1989; Humphries et al., 1999). Recording total number of scent markings, regardless of location, has revealed different responses to different test scents (Hurst et al., 2001; Nevison et al., 2003).

Otters were observed to make direct contact with spraints with their noses suggesting non-volatile compounds may be involved in communication. Countermarking was observed much more in these experiments compared with preliminary experiments where direct contact was

prevented. Little or no countermarking has been observed in other species when direct contact with scent marks is prevented (North American river otters, Rostain et al., 2004; mice, Nevison et al., 2003). Nevison et al. (2003) conclude this is because the ownership signal is involatile. In the preliminary experiments (data not presented here), no otter spent more time investigating the control rather than test scents, suggesting that at least some olfactory information is detected through volatile components of the spraint. In the experiments presented in this chapter, investigation was characterised by otters sniffing the air leading to the spraint and making direct contact with the scent, sometimes followed by more sniffing of the air. These behavioural observations suggest both volatile and non-volatile components are used; perhaps volatiles allow scents to be located and non-volatiles contain information about the sender. In this scenario volatile compounds still play a vital role as associations may be learned between volatiles and non-volatiles allowing future detection of the information from a distance (Hurst, 2009).

The results of this chapter suggest differences in spraint odour due to the sex and progesterone level of the depositor may be detected by otters. Behavioural responses of otters suggest 4 day old scent is of equal interest to spraint deposited within 24 hours. Further, experimentation is needed to determine for how long spraints remains of olfactory interest to otters. The unavoidable small sample size limit generalisation of these findings and prevented the investigation of sex differences in responses which needs further investigation. Importantly this chapter provides evidence of a role for non-volatile substances in otter scent communication and that otters learn where scent messages are likely to be located.

Chapter 3
Method development and validation experiments

3.1. Introduction

To determine differences in otter scent relating to otter biology, and test whether spraint volatile organic compounds (VOCs) can be used effectively to identify otters, it is first necessary to optimise sampling and analysis of VOCs and to ensure that other ecological variables, such as diet, do not confound the target variables, such as age, sex or individual identity. Method development or optimisation experiments in chemical ecology are rarely reported in the published literature; where present, they are often briefly referred to as preliminary experiments with no indication what other methods were tested. An exception to this is Zomer et al. (2009) who reported testing five solid-phase microextraction (SPME) fibres and then selected the fibre that recovered the greatest number of VOCs for further analysis. The greatest number of compounds may not, however, be the best measure as different fibres sample different types of compounds (examples in Table 3.1). In other subject areas of analytical chemistry, whole journal articles may be dedicated to method development. In analysis of textile VOCs for example, Zhu et al. (2009) report the investigation of headspace equilibrium temperature and time, extraction time of SPME, and thermal desorption temperature and time.

There are various methods available in chemical ecology to sample VOCs from mammal scents, for example direct headspace sampling, solvent extraction of liquid samples, adsorbent traps with solvent elution or the relatively recently developed SPME. Previous attempts to investigate the nature and function of *Lutra lutra* scent used solvent extraction (Trowbridge, 1983; Bradshaw et al., 2001). The main disadvantage of solvent extraction is that compounds are diluted and concentrations of some may be reduced to below detection limits.

SPME allows solvent free sampling. The technique collects VOCs by partitioning, or portioning and adsorption into the coating of fibres which are then inserted into a gas chromatography (GC) injector for thermal desorbtion of the VOCs that are then carried by the

gas into the column. The technique is relatively simple compared to other methods and generally involves a shorter sampling time than direct headspace sampling (sampling the gas above a solid or liquid sample). SPME can be used for thermal desorbtion and GC, or desorption into high performance liquid chromatography (HPLC). SPME can be used to collect volatiles from the headspace above samples, even very small amounts of secretion. GC is the method of choice for analysis of complex mixtures of volatile or semi-volatile compounds (Heath and Dueben, 1998) and is the most appropriate technique to use with SPME. GC can be coupled with mass spectrometers (GCMS) to provide structural information to identify unknown compounds.

To date SPME has not been used in mustelid chemical ecology research (Table 1.1); the current exploration therefore presents a significant advancement in chemical analysis of mustelid scent. To optimise the number of different VOCs sampled, and the quality of the signals, it is necessary to test the effects of various experimental conditions. For example, the fibres available for SPME differ in thickness and in the type of coating, which select for compounds of differing polarities and volatilities. In this chapter a series of experiments are presented that were used to optimise the experimental conditions for analysis of VOCs from otter anal scent gland secretion, and spraint using SPME and GC-MS. Specifically, the type of fibre and exposure time are tested as well as the reproducibility of results.

To obtain a sufficient number of samples from known individuals (for both this chapter and Chapter 4), it was necessary to use gland secretion from dead otters. There are not enough captive Eurasian otters and direct observation in the wild is impractical. To obtain sufficient sample numbers, anal glands were collected from carcasses over several years. As this necessitated storage, it was necessary to test the impact of prolonged freezer storage. Although research on other species suggests the validity of sampling scent from carcasses (e.g. Rosell and Sundsdal, 2001, found similarities in scent taken from dead beavers and samples collected in the wild), it is unclear whether post-mortem decay, or subsequent storage of samples prior to analysis, might affect the VOCs eluted. Otters sent to Cardiff University for post-mortem examination are in varying states of decay and often the time elapsed between death and collection is unknown. It was therefore necessary to test the impact of the state of decay on scent gland VOCs.

This thesis contributes to an ultimate aim to use chemical analysis to identify parameters about otters from spraint samples collected in the wild. Categorical differences in scent are established using anal scent gland secretions from carcasses (Chapter 4), but spraint is a combination of anal gland secretion and faeces. Therefore in this chapter I examine the VOCs from faeces and anal gland secretion to assess how each may contribute to spraint scent. The most important demographic characteristics to distinguish using scent are examined in detail in other chapters, for example, age, sex (Chapter 4) and individuality (Chapter 5), however other variables may contribute the scent variability and could complicate the detection of target variables. There is evidence from other species of scent being associated with diet; it may be used, for example, to assess male quality (Giaquinto et al., 2010) or interspecifically to assess predation risk (Mathis and Smith, 1993). In this chapter I present a preliminary experiment that tests how likely it is that diet will confound the differentiation between target demographic variables.

This chapter, therefore, addresses a number of method development and validation experiments:

- Development of volatile sampling method focusing on SPME fibre selection and exposure time.
- Anal gland storage
- Change in scent gland secretion inside a decaying carcass
- Sample type: anal gland secretion and faeces
- The effect of diet on otter scent

3.2. Methods

3.2.1. Sample collection

Otters found dead in England and Wales are collected for post-mortem examination by Cardiff University Otter Project. Most otter carcasses are frozen, then couriered to Cardiff University, a small minority are sent without freezing. Carcasses are stored at approximately -20°C until post-mortem examination. The carcasses are categorised as fresh, slightly

decomposed, moderately decomposed or very decomposed, according to signs of autolysis. Unless otherwise stated, all samples examined for this study were from fresh otters.

For post-mortem examination carcasses were defrosted for approximately 48 hours at room temperature. Anal glands were removed at post mortem, foil wrapped and stored in ziplock bags at -20°C. Anal glands have been retained routinely since 2000 resulting in a frozen archive of over 600 samples. Generally, the anal glands were kept intact still attached to the anus. Data regarding the location where the otter was found, sex, age group, reproductive status, post mortem details and several organ measurements were recorded on a Microsoft Access ™ database.

3.2.2. Development of volatile sampling method

Preliminary experiments tested the use of dynamic headspace with charcoal (Alltech Associates Ltd) or Poropak (P/N# VCT-1/4X3-POR filled with 10mg Poropak) filters to sample VOCs from otter anal gland contents. Dynamic headspace was deemed unsuitable because sample preparation took approximately 4 hours and the resulting chromatograms contained very few peaks and were at low intensity. Selection of VOC sampling method, therefore, focused on SPME.

a) Fibre selection for number and size of chromatogram peaks

SPME fibres were compared to establish which fibre collected the most VOCs from otter anal gland contents. A polydimethylsiloxane 100μm non-bonded (PDMS) fibre and a polyacrylate 85μm bonded fibre were exposed for 60 minutes to the anal gland contents of one adult male otter. Preliminary experiments showed that exposure times of 10 and 20 minutes gave very few chromatogram peaks. The anal gland contents from another adult male otter was sampled with a PDMS fibre and a StableFlex Divinylbenzene/Carboxen/PDMS 50/30μm bonded (DVB/CAR/PDMS) fibre using the same methods.

b) Selection of SPME exposure time

A DVB/CAR/PDMS fibre was exposed to the contents from the anal glands of one otter for 15 minutes, 30 minutes and 1 hour to select the optimum exposure time (this fibre was tested because of the results of the previous experiment in Section 3.2.2.a).

c) Fibre selection for optimal separation

To ensure the best fibre was selected, optimal separation was measured in addition to the number and size of chromatogram peaks. The fibre that obtains the greatest number of chromatogram peaks may not absorb the compounds involved in discrimination between target variables. As the aim of this research is to discriminate otter samples based on otter biology, 30 samples were analysed to compare which fibre could be used to best discriminate between adult male, adult female, sub-adult male, sub-adult female, juvenile male and juvenile female otters. Otters were assigned to the given age-groups (adult, sub-adult, and juvenile) at post-mortem, based on body size and reproductive status (Chadwick 2007). The fibres tested were DVB/CAR/PDMS and PDMS (the best and second best according to the experiment described in Section 3.2.2.a). Stratified random sampling was used to limit bias but ensure samples originated from these six groups and from different geographical regions. Pregnant and lactating females were excluded, as were females with suspected but unconfirmed pregnancy. To minimise bias, the order that the fibres were exposed to each sample was alternated. The optimum exposure time for DVB/CAR/PDMS selected from the previous section was used (45min) and 60 minutes for the PDMS fibre (given results of preliminary experiments detailed in Section 3.2.2a).

d) Method reproducibility

To test the reproducibility of the analytical data three replicates of five otter anal gland samples were analysed. The DVB/CAR/PDMS fibre was exposed to samples for 45 minutes.

3.2.3. Anal gland storage

Ten samples from otter carcasses stored for eight years (long storage) and six samples collected from carcasses stored for 6-10 months (short storage) were analysed. For consistency, samples were all from fresh adult male otters found in Wales or border counties (hence the difference in sample number between the two time periods). Once expressed from the glands, secretion samples were weighed and the colour of the secretion was categorized by comparison to a colour chart, as light, medium or dark brown, mix of brown and white, or white. The smell of the secretion was categorized as faecal, sickly sweet, sweet, old oil, other unpleasant, or other not unpleasant. Colour and smell were recorded because differences in these parameters may allow a cheap and easy way to identify suitable samples in the future.

3.2.4. Scent gland secretion inside a decaying carcass

The anal scent glands, anus, short section of lower gut (up to 10cm) and surrounding tissue and pelt were removed from the carcass of an adult male otter approximately within 24 hours of death and kept at room temperature, exposed to the air. It was not possible to retain the glands within the whole carcass as many of the other organs are used in other research, for example pollutant levels in liver. A few drops of secretion were expressed manually from the anal scent glands into an SPME vial once per day for five days. The VOCs were sampled within one hour of collection. This was conducted only on one otter because there were no other suitable otters during the period of sampling.

3.2.5. Sample type: anal gland secretion and faeces

During post-mortem examinations, a small amount (approx. $1cm^3$) of faeces was collected from the alimentary canal just prior to the anus (therefore before contact with the scent glands) from 10 otters. The faeces were stored and defrosted in the same way as anal glands. Faeces and anal gland secretions were analysed separately. The gland secretion was then poured over the faeces to imitate how faeces and gland secretion is usually deposited as spraint by otters. This combination of faeces and gland secretion was then also sampled and analysed.

3.2.6. Diet

To investigate if diet is likely to confound the identification of target variables (age, sex, individual identity) in otter scent, five otters were selected whose stomachs contained only amphibian remains and another five whose stomachs contained only eel remains. These two prey types were selected because they are from different vertebrate classes and are therefore particularly different from each other. If diet differences in scent are not found with these, it is unlikely that they would exist for more closely related prey types, for example between eels and other fish species. Prey remains from the stomach contents of dead otters were identified as part of a separate study on otter diet. Stomach contents were placed into a beaker with biological washing powder until all soft remains were digested. The remaining hard parts were cleaned and sorted, and prey species identified using keys (Conroy et al., 2005). From these 10 otters, faeces, anal gland secretion, and a mixture of the two (as detailed in Section 3.2.5) were analysed. Following scent analysis faecal samples were examined to

confirm prey remains where possible (i.e. where there were identifiable hard parts). It was possible to confirm prey remains in two out of five cases for each prey type.

3.2.7 Chemical analysis

Prior to analysis, anal glands were defrosted overnight in a refrigerator at approximately 4°C. The complete contents of both glands were expressed manually into one 10ml SPME glass vial (Supelco), sealed and weighed. The anal gland contents were then left to equilibrate in the vial at room temperature for approximately 1 hour. Supelco SPME fibres were used to sample VOCs eluting from anal gland secretions, by exposing the fibre to the headspace in the vial above the sample. All three fibres tested (Table 3.1) were conditioned according to manufacturer's recommendations and reconditioned if the fibre had not been used for several hours. An analysis of the fibre not exposed to any sample was conducted at least every sixth sample to detect any contamination or deterioration of the fibre, and fibres were replaced when damaged. Sample vials were placed in a water bath to ensure a consistent temperature during sampling set at 20^{o}C for the first experiment (Section 3.2.2.a) and 30^{o}C in all other experiments. The water bath temperature was increased because applying heat can increase the number and concentration of analytes released from solid samples.. The time the fibre was exposed to the sample varied between experiments (see individual experiments above).

Table 3.1. Solid Phase Microextraction (SPME) fibres tested for efficacy of sampling volatile organic compounds (VOCs) from otter scent.

Stationary phase and film thickness	**Polarity/ function**	**Conditioning (and reconditioning) temperature** *within manufacturer's recommendations*	**Desorption temperature**
Polyacrylate 85μm bonded	Polar/ Partitioning	300°C (220°C)	220°C
Polydimethylsiloxane (PDMS) 100μm non-bonded	Non-polar/ Partitioning	250°C (220°C)	220°C
StableFlex Divinylbenzene/Carboxen/PDMS (DVB/CAR/PDMS) 50/30μm bonded. 2cm without spring.	Bi-polar/ Adsorbent, partitioning	270°C (260°C)	260°C

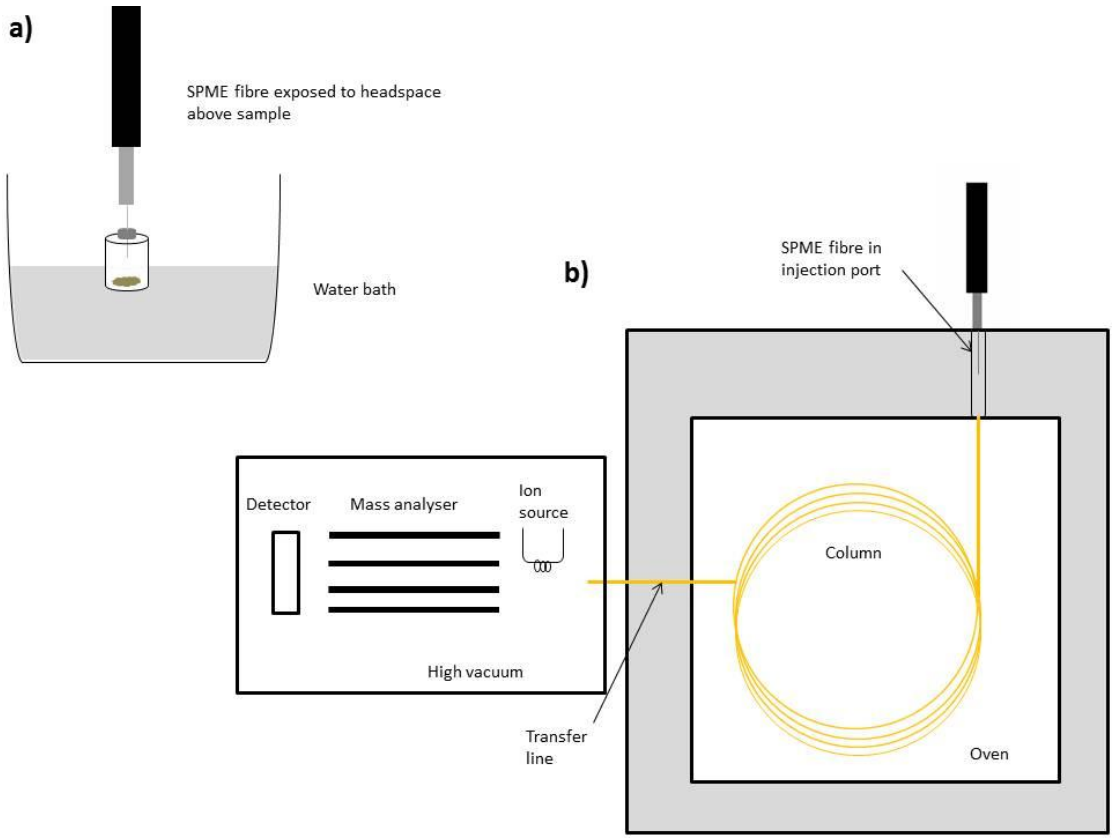


Figure 3.1. Diagram of (a) sampling and (b) analysis equipment used in the analysis of otter (*Lutra lutra*) scent samples. Solid phase microextraction (SPME) was used to sample volatile organic compounds from the headspace above samples and then analysed with gas chromatography mass spectrometry (GCMS).

All samples were analysed using GC-MS (Agilent 6890N/5973N). Following exposure to sample headspace, fibres were immediately injected manually into the GCMS injection port and desorped for 2 minutes in the split/splitless injection port fitted with an SPME liner (Supelco) in splitless mode. The GC was fitted with a 30 m, 0.25 mm I.D., 0.25 µm Zebron 5ms capillary column (Phenomenex) with helium as carrier gas at constant pressure (13.4 psi). The injection port temperature was set according to the manufacturers recommended temperature for each fibre type (Table 3.1). The oven programme varied between experiments. Oven programmes are detailed in Table 3.2. Temperature of transfer line, ion source and analyser of MS were set to 310°C, 230°C and 150°C, respectively. Mass spectra were recorded without solvent delay from m/z 35 to 550.

After every 4-6 samples, 0.2µl of an external hydrocarbon standard (MA EPH Aliphatic Hydrocarbon Standard [Restek] diluted 1:50 with n-hexane [Fisher Scientific AnalR]) was injected using an automatic liquid injector to check the performance of the GC-MS and for calculation of retention indices. This allows standardisation of retention times. Compounds were provisionally identified (minimum match factor between the deconvoluted component and the library spectra of 80%) and quantified using Automated Mass Spectral Deconvolution and Identification System (AMDIS) version 2.65 and the NIST Mass Spectral Library Version 2.0 (2005). Both mass spectral data and retention indices were used by AMDIS in identification.

Table 3.2. GCMS oven programmes tested for efficacy of analysing volatile organic compounds (VOCs) from otter scent. Many peaks with low retention times co-eluted with Oven programme A so changes in the oven programme were made to prevent this.

Oven Programme	Start temp. hold	1st increase	2nd increase	Final temp. hold	Total run time (min)	Experiments
A	40°C for 2.5 min	5°C min^{-1} up to 300°C		300°C for 5 min	59.5	Fibre selection for peak number and size
B	40°C for 2.5 min	2.5°C min^{-1} up to 140°C	16°C min^{-1} up to 300°C	300°C for 4 min	56.5	Exposure time selection
C	40°C for 2.5 min	1.5°C min^{-1} up to 100°C	20°C min^{-1} up to 300°C	300°C for 4 min	56.5	All others

3.2.8 Data analysis

Peaks with a retention time below 2 minutes were not included in the analysis because signals with retention times close to the hold-up time of the system are not measurable with sufficient accuracy. As an internal standard was not used, absolute values could not be measured; instead the relative contribution of each peak to the overall scent profile was calculated (i.e. data were normalised). Data were also standardised across compounds to

ensure compounds were given equal weight in the analysis, irrespective of their size. Zero values were replaced with half the value of the lowest intensity compound measured.

There is no consensus on the best way to analyse scent data, therefore a number of statistical tests were tried as part of the method development. Principal Components Analysis (PCA) was conducted to select the fibre selection that enabled optimal separation (Section 3.2.2.c). PCA and hierarchical cluster analysis were used to examine method reproducibility (Section 3.2.2.d). To examine the effect of anal gland storage time (Section 3.2.3.) on sample weight and total chromatogram area, 2-tailed independent samples t-tests were conducted. A Mann-Witney U test was conducted to compare the number of chromatogram peaks between short and long storage. Euclidean distance was calculated between each pair of samples from the scent gland secretion in the decaying carcass.

PCA was conducted to visualise differences in scent between samples types (Section 3.2.5.) and otter diet (Section 3.2.6.). Differences in chemical profiles between sample groups (sample type or otter diet) were tested using Permutational Multivariate Analysis of Variance (PerMANOVA), with a Euclidean distance matrix and 999 permutations. All data analysis was conducted in SPSS (version 16.0), apart from the PerMANOVAs that were performed using the function Adonis in the vegan package (Oksanen et al., 2011) in R (version 2.13.1, R Development Core Team, 2011). There is no post-hoc test available for PerMANOVA using Adonis so individual models were performed to investigate differences between categories of a variable (sample type or diet). The p value for PerMANOVA is derived from the number of permutations that result in an F statistic equal to or greater than the observed F value. PerMANOVA has the advantage over other techniques such as ordination because it uses all of the scent profile rather than a subset of the data and is thought to be more robust than analysis of similarity (ANOSIM) or multiple response permutation procedure (Oksanen et al., 2011). It was developed for analysis of ecological data which often has more variables than sampling units and skewed distributions of abundances of species (Anderson 2001), which is similar in structure to the scent data collected here.

3.3. Results

3.3.1. Development of volatile sampling method

a) Fibre selection for number and size of chromatogram peaks

With the same adult male otter anal gland sample 98 chromatogram peaks were found using the PDMS fibre and only 71 with the polyacrylate fibre. The total chromatogram area was 1.01 times larger using the PDMS fibre (Figure 3.2). With another adult male anal gland sample, 261 chromatogram peaks were found using the DVB/CAR/PDMS fibre and only 56 using the PDMS fibre. The total chromatogram area was 9.19 times larger (Figure 3.3).

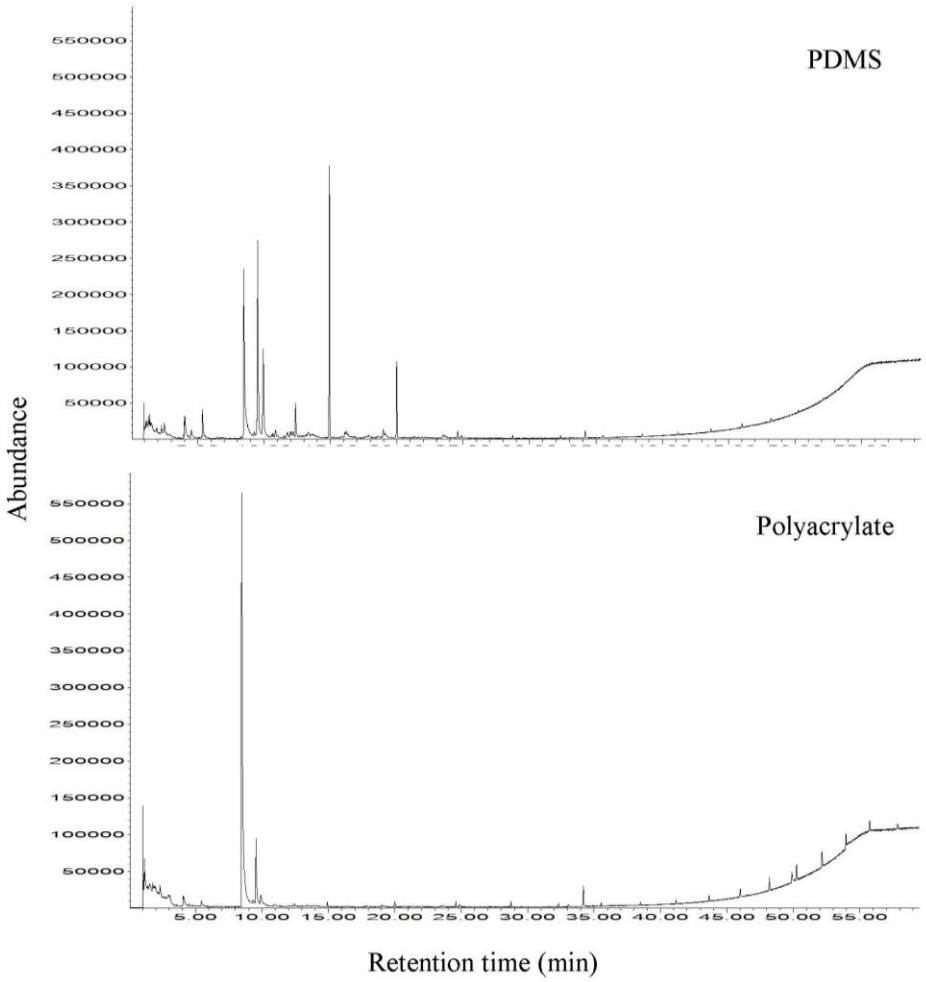


Figure 3.2. Total ion chromatograms from GC-MS analysis of VOCs from the anal gland contents from one adult male otter sampled using PDMS and polyacrylate solid phase microextraction fibres.

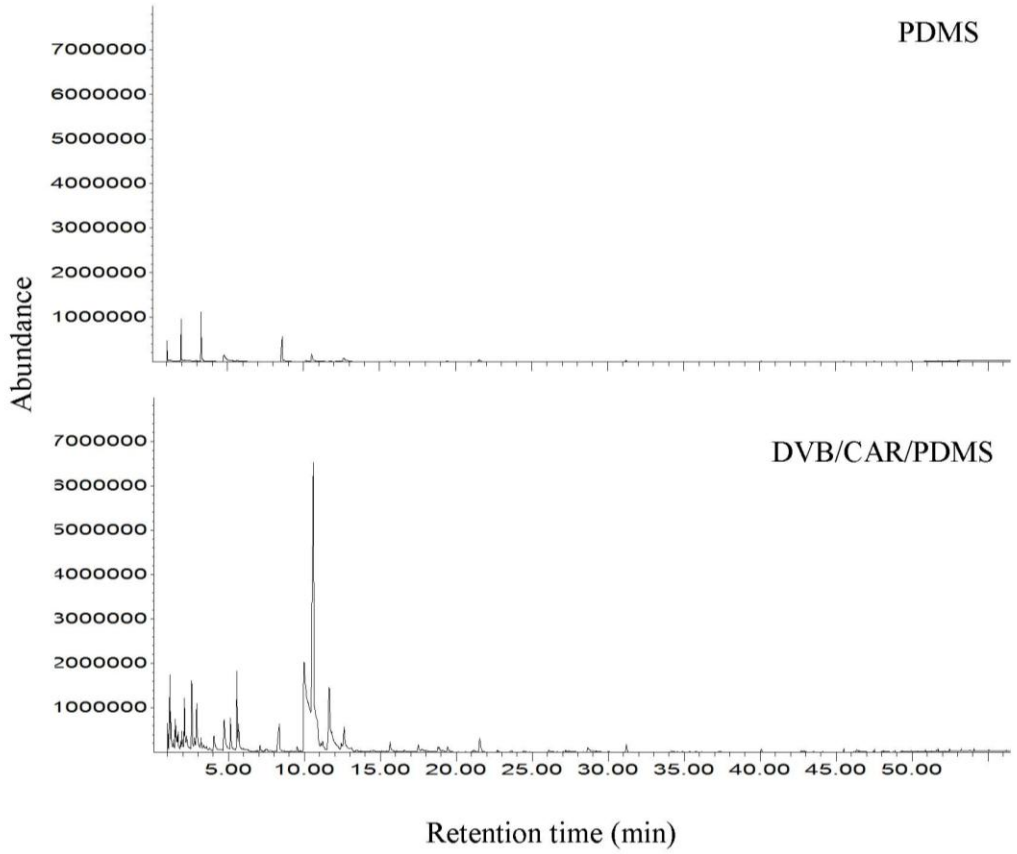


Figure 3.3. Total ion chromatograms from GC-MS analysis of VOCs from the anal gland contents from one adult male otter sampled using PDMS and DVB/CAR/PDMS solid phase microextraction fibres.

b) Selection of SPME exposure time

More peaks were obtained with longer exposure times; 139 peaks with 15 min, 153 with 30 min, 165 with 60 min (Figure 3.4). Examination of the AMDIS identification of components showed few differences between 30 and 60 minutes. The optimum exposure time (a compromise to enable the analysis of a high number of samples whilst still sampling a high number of compounds) for the DVB/CAR/PDMS fibre is between 30 and 60 minutes so an exposure time of 45 minutes was used for subsequent samples.

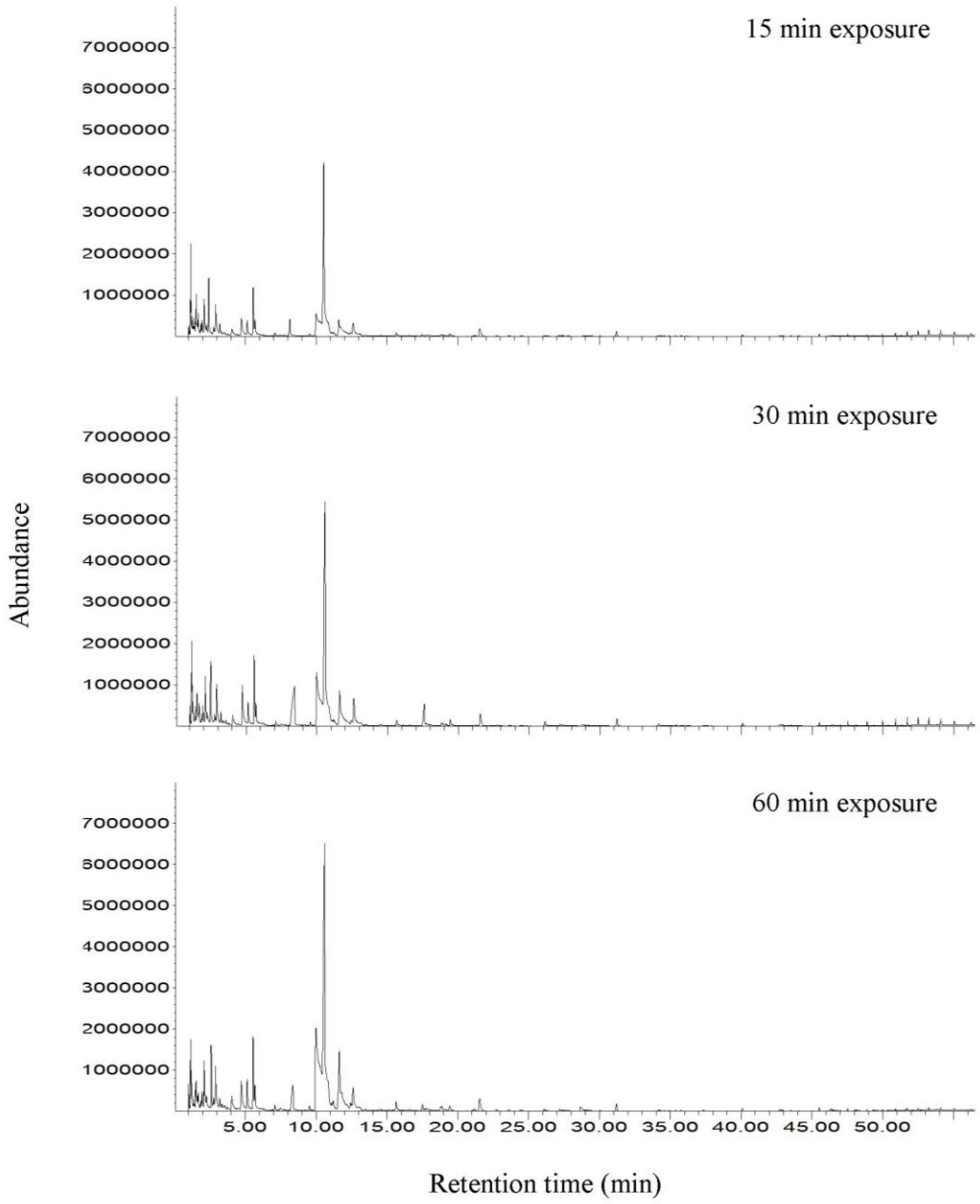


Figure 3.4. Total ion Chromatograms from GC-MS analysis of VOCs from the anal gland contents from one adult male otter sampled using a DVB/CAR/ PDMS solid phase microextraction fibre exposed for different lengths of time.

c) Fibre selection for optimal separation

The PDMS fibre appeared to allow separation of juvenile females from sub-adult and adult females (apart from one sample), but no discrimination between other groups. Despite an additional 15 minutes exposure, the PDMS fibre did not provide good separation between sample types. The DVB/CAR/PDMS fibre gave better discrimination with all juveniles

separating from adults. Additionally, male sub-adults grouped with juvenile samples, and female sub-adults grouped with adult samples (apart from one sample) (Figure 3.5). The DVB/CAR/PDMS fibre was therefore selected for subsequent analysis. The VOCs recovered from these 30 anal gland samples using the DVB/CAR/PDMS fibre were used to create a custom library (referred to as the gland library and detailed in Appendix 1) in AMDIS that is used in Chapter 4 to analyse other samples. Any compounds with a match factor of <80% were individually examined and either added to the library as potential identifications (e.g. those with match factor 60-80%) or named as unknown and given a number.

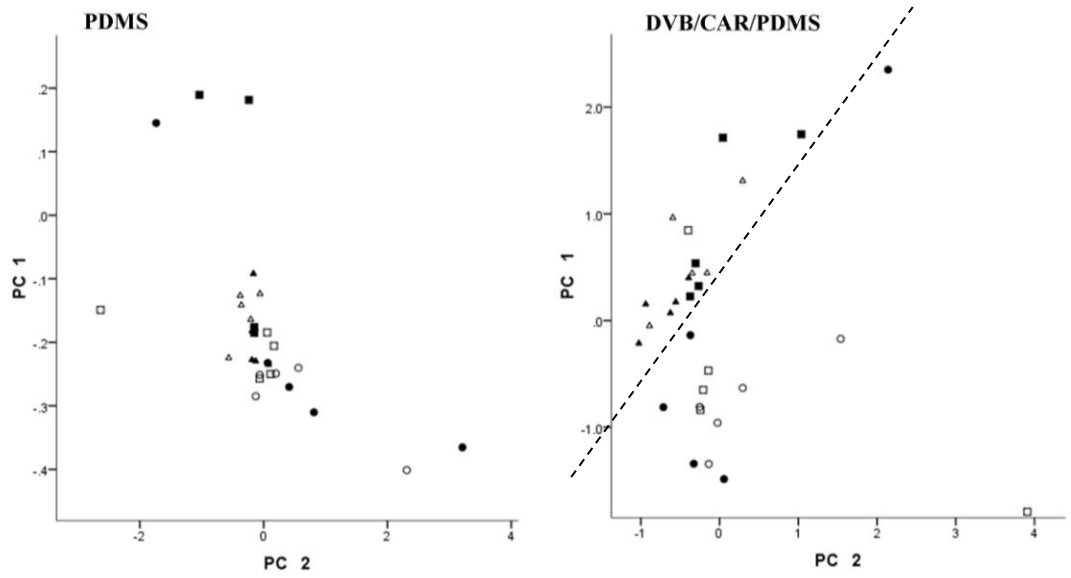


Figure 3.5. Principal Components Analysis plots from the chemical analysis of otter anal gland secretions using two different SPME fibre types. Filled symbols =male, open symbols=females, ○=adults, □=sub-adults, Δ=juveniles. Dashed line indicates differentiation of juveniles and male sub-adults from all adults and sub-adult females.

d) Method reproducibility

The first four principle components, explaining 64% of the total variance of 382 VOCs found in the otter scent gland secretion, were used in a hierarchical cluster analysis, using square Euclidean distance as the similarity measure. Repeats of the same sample clustered (Figure 3.6), indicating the reliability of the method developed for sampling otter scent VOCs.

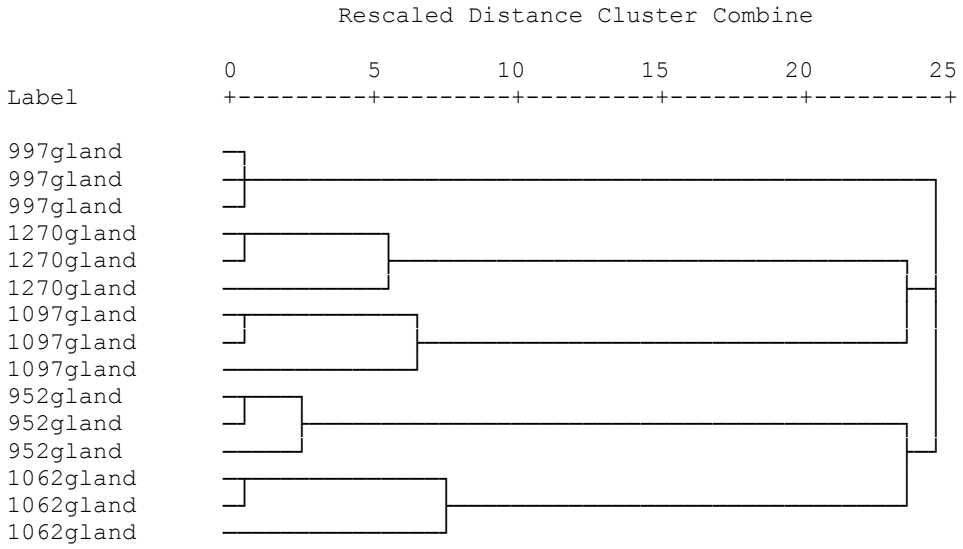


Figure 3.6. Clustering of repeated samples of VOCs from otter anal gland secretions. The numbers before gland indicate the otter reference number.

3.3.2. Anal gland storage

There was no apparent difference in colour, thickness, strength or nature of smell to the human observer between samples stored frozen for a short (6-10 months) or long (8 years) period of time. There was no significant difference in weight (g) between samples stored for a long period (mean= 1.53g, SD= 1.02) and those stored for a short period (mean=2.36g, SD=0.81; t (14) =-1.683, p=0.114). Total chromatogram area did not differ significantly between samples stored for a long (mean=1.91 x 10^9, SD=1.153 x 10^9) or short (mean=2.26 x 10^9, SD=5.57 x 10^8; t (14) =-0.683, p=0.506) period of time. Neither was there a significant difference in the number of chromatogram peaks between samples stored for a long (mean=176.00, SD=35.827) or short (mean=161.33, SD=31.430; Mann-Whitney U=21, p=0.328) period of time.

3.3.3. Scent gland secretion inside a decaying carcass

The scent profile of gland secretion taken from the decaying scent glands became more complex over five days; the number of compounds increased from 40 through 55, 74, 64 to 85 on Days 1-5 respectively. Dimethyldisulfide and trimethyldisulfide were not detected until Day 3 and then increased on Day 4 and again by Day 5. Calculation of Euclidean distances between samples revealed that the scent gland secretion became more dissimilar over time

(Table 3.3). Samples selected for subsequent analysis were therefore taken only from otters deemed to be fresh at time of collection.

Table 3.3. Dissimilarity, based on Euclidean distances, between VOC profiles of otter anal scent gland samples taken from a dead otter over five days.

	Day 1	Day 2	Day 3	Day 4	Day 5
Day 1	.000	.134	.181	.307	.309
Day 2	.134	.000	.082	.183	.197
Day 3	.181	.082	.000	.139	.136
Day 4	.307	.183	.139	.000	.077
Day 5	.309	.197	.136	.077	.000

3.3.4. Sample type: anal gland secretion and faeces

From the ten faeces samples, 61 extra compounds were found that were not in the gland library (Appendix 1). Most of these compounds occurred infrequently (1-3/10), only two compounds were found more commonly; pyrrole (5/10 faeces samples) and 3-methyl-1-butanol (5/10). Gland and 'artificial spraint' samples were more complex than faeces (Figures 7 and 8). There was a significant difference in the number of compounds between faeces samples, gland samples and 'artificial spraint' (means= 33, 50, 42 respectively; Kruskall-Wallis, df=2, Chi-Square=12.75, p=0.002).

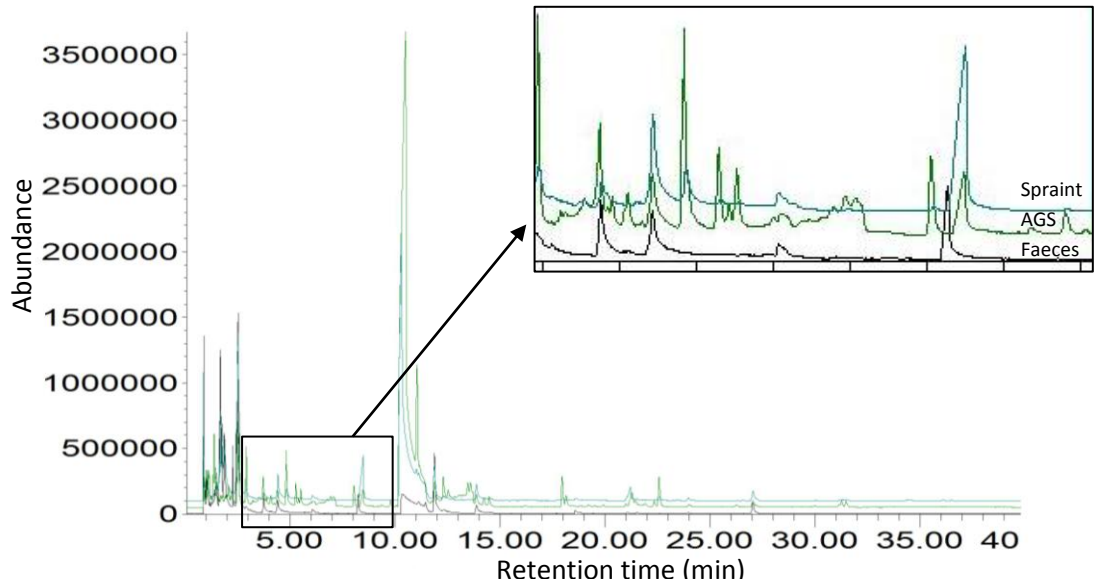


Figure 3.7. Overlain Total Ion Chromatograms (TIC) from GC-MS analysis of VOCs from the — faeces, — anal gland secretion (AGS), and — artificial spraint (AGS and faeces combined) of an otter. A section is enlarged to show higher complexity of AGS.

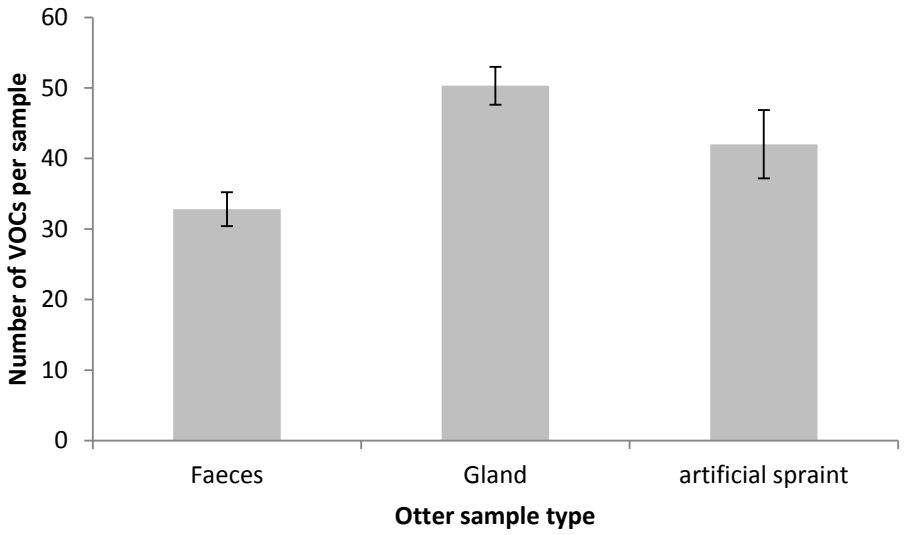


Figure 3.8. Number (mean ± standard error) of volatile organic compounds (VOCs) differed significantly between three different sample types taken from ten otters (Kruskall-Wallis, p=0.002).

The first two principal components (PCs) explained only 21% of the total variance of the 359 provisionally identified compounds across the 30 samples. Anal gland samples were more variable on PC1 and PC2 than faecal samples (Figure 3.9). Two outliers were identified from PCA and these were removed before PerMANOVA was performed. Scent profiles differed significantly between sample types (F_{pseudo} (2, 27) =1.42, R^2=0.10, p=0.001). Faeces scent was significantly different from anal gland scent (F_{pseudo} (1, 18)=1.86, R^2=0.10, p=0.001) and 'artificial spraint' scent (F_{pseudo}(1, 18)=1.35, R^2=0.07, p=0.001). Anal gland secretion was significantly different from 'artificial spraint' (F_{pseudo}(1,18)=1.25, R^2=0.07, p=0.015).

3.3.5. Diet

None of the sample types differed in PC1 by diet (Figure 3.10; PC 1 explained 11% of total variation). There was no overal significant difference in scent between the two diets (F_{pseudo} (1,28)=1.10, R^2=0.04, p=0.12). There was no significant difference between otters that had only eaten eels and otters that had eaten only amphibians in either faecal scent (F_{pseudo}(1, 8)=0.95, R^2=0.11, p=0.72), anal gland secretion scent (Fpseudo (1,8)=0.94, R^2=0.11, p=0.74) or artificial spraint (F_{pseudo} (1,8)=0.93, R^2=0.10, p=0.93).

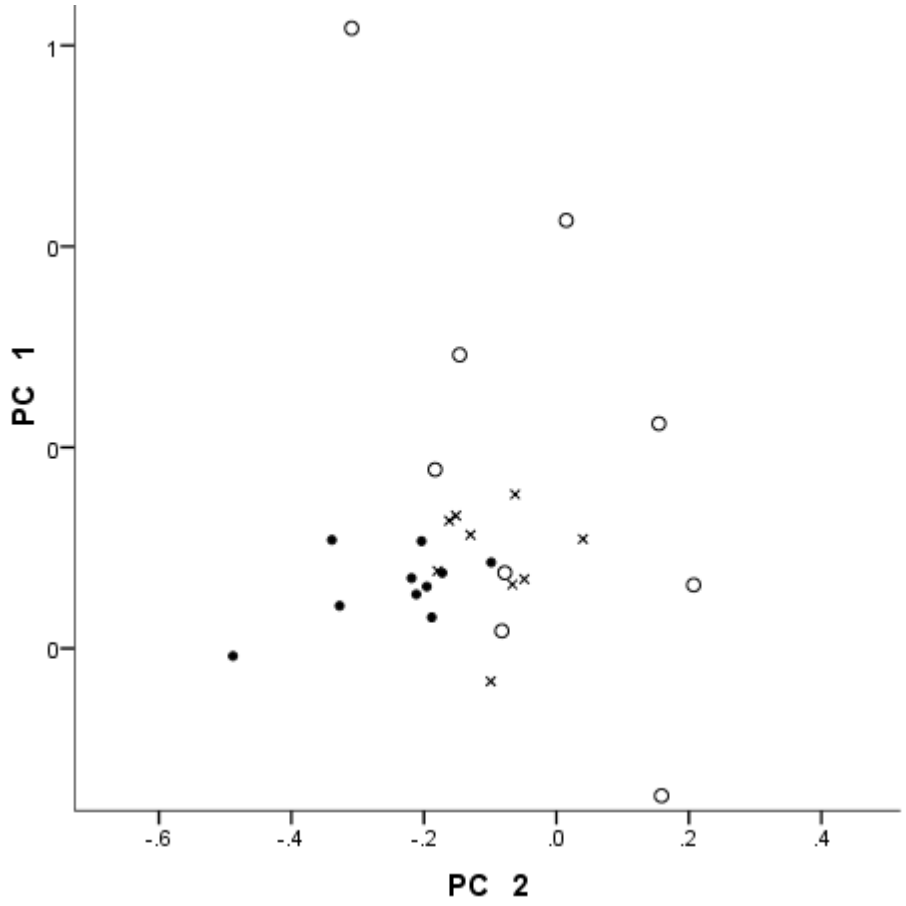


Figure 3.9. PC 1 and PC2 from chemical profiles of three different sample types from otters (n=10) showing some differentiation between sample types (●= faeces, ○= gland secretion, x= artificial spraint).

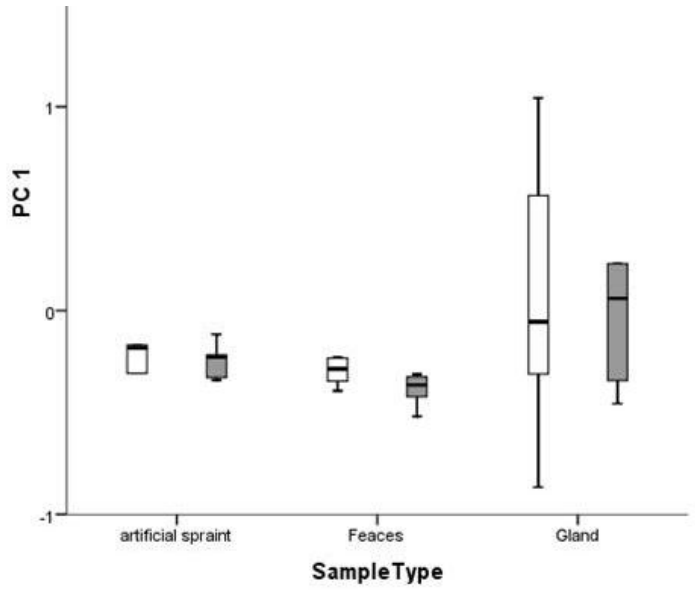


Figure 3.10. PC1 scores of scent analysis by sample type and otter diet. Filled boxes indicate that only eel remains were found in the otter stomach and unfilled boxes indicate that only amphibian remains were found in the otter stomach.

3.4. Discussion

Method development and optimisation is necessary to increase the likelihood that differences in scent relating to target variables, in this case those associated with otter identity, are found. Although a key aspect of analytical chemistry, chemical communication studies lag behind related disciplines in their reporting of method development. Here, on the basis of the multiple experiments detailed above, two sets of recommendations are suggested below. Firstly, recommendations for the development of repeatable methods appropriate to scent sampling and analysis in ecological studies (Section 3.5.1), and secondly, the optimum methods to analyse VOCs from otter scent samples (Section 3.5.2; as used in subsequent chapters unless otherwise stated).

The methods developed to collect otter anal gland secretions, sample VOCs eluting from those secretions and analyse those VOCs were found to be repeatable and so were used in subsequent analyses. The DVB/CAR/PDMS fibre was considered better than the PDMS and polyacrylate fibres based on the number and total abundance of compounds, as well as preliminary discrimination between age and sex groups of otters. This is the same fibre type that Zomer et al. (2009) found recovered the most analytes from mouse urine, however, the greatest number of compounds should not be relied upon to provide the best discrimination, as different fibre types absorb different types of compounds. Although solvent extraction has historically been popular for the analysis of animal scent marks, SPME offering solvent free analysis was developed in the 1990s and is becoming more commonplace (although as yet there is no published research on mustelids using this technique). The PDMS fibre has been used successfully for porcupine (Massolo et al., 2009) and giant panda (Hagey and Macdonald, 2003) scent samples, and the DVB/CAR/PDMS fibre, selected as the best fibre for otter scent in this chapter, has also been used successfully to distinguish between the scent of canid species (Burnham et al., 2008) and various variables from mouse urine (Schaefer et al., 2010; Kwak et al., 2008). The most appropriate fibre type is likely to vary between species and studies should report, at least briefly, which fibre types were tested, otherwise the question remains if other fibre types may find differences. For example, Carboxen fibres have been used to distinguish between individual spotted hyaenas but no differences relating to age or relatedness were found (Burgener et al., 2009). Perhaps these differences could be found with other fibre types but the authors do not state if other fibres were tested.

The exposure time selected in this chapter for otter scent is within the range of these studies (5 – 60 minutes). The exposure time selected (45 minutes) was a compromise; although additional exposure time did obtain additional peaks, the increase in peak number was not as large as initial increases in exposure time (between 15 and 30 minutes) and the additional time would have significantly increased the amount of laboratory time needed to complete all experimental work for subsequent samples. After injection into GCMS, both Schaefer et al. (2010) and Burnham et al. (2008) performed additional cleaning of DVB/CAR/PDMS fibres to reduce carry over. In subsequent chapters, fibres were injected into another GC injection port at 260^{o}C for 10 minutes to clean off any remaining VOCs.

Although based on samples from only one otter, analyses of gland contents taken from a decaying otter over a 5 day period clearly showed a change in composition over time. Dimethyldisulfide and trimethyldisulfide in gland secretion increased with time from death. Low-molecular sulphur containing VOCs are known to be produced by decaying mice (Kalinova et al., 2009) and dimethyldisulfide has been reported as the highest concentration of all VOCs from decaying human bodies (Statheropoulous et al., 2005). Dimethyldisulfide and dimethyltrisulfide are known to attract carrion beetles to pitfall traps (Podskalská1 et al., 2009) because of this association with decay. Samples selected for subsequent analysis were therefore taken only from otters deemed to be fresh at time of collection.

Spraint is a combination of anal gland secretion and faeces. The results presented show anal gland secretion and faeces differ in odour and when combined this also differs from both of the sample types alone. Faecal samples contained compounds that were not present in the gland library. The two frequently occurring faecal VOCs not in the gland library were pyrrole and 3-methyl-1-Butanol. Pyrrole has been found in meats (Machiels et al., 2003) indicating the digestive tract of a carnivore can produce similar compounds, however it has also been found in very dissimilar samples, for example coffee (Sanz et al., 2001). Faecal samples varied less than anal gland secretions; faeces may be used as a carrier for scent signals rather than being part of the signal itself. The results in this chapter indicate that gland scent is more variable and therefore it could be suggested that gland scent is more likely to contribute towards communication. Faeces may, however, contain differences in scent relating to the

target variables, therefore, subsequent analysis of spraint (Chapter 5 and 6) did not focus exclusively on compounds found in anal gland secretion.

Odours produced by animals are a complex interaction of internal physiological and metabolic state, including diet (Wyatt, 2003). The results presented here, based on two markedly different prey types, suggest that diet does not influence the VOCs eluting from faeces or anal gland secretion. This is surprising as there is behavioural evidence the ability to discriminate between scent marks from conspecifics fed on different diets (Hobbs et al., 2008; Hobbs and Ferkin, 2011; Kwak et al., 2008). Chemical evidence for dietary differences also exists, for example, diet affects the faecal concentrations of short chain fatty acids (acetate, propionate and total concentrations) that are markers of microbial metabolism in dogs (Martineau and Laflamme, 2002). Diet also influences VOCs from mouse urine (Kwak et al., 2008; Schaefer et al., 2010) although both of these used some form of peak selection, rather than including all of the peaks in analysis as in the current study. Peak selection is very susceptible to Type 1 error. Apart from this difference in statistical analysis method, the analysis conducted here is expected to be similar to these other studies that have tested diets with different levels of protein (Hobbs et al., 2008; Kwak et al., 2008) or fat (Schaefer et al., 2010). Although I only tested two prey types, eels, a preferred food source for otters, provide much higher kJ per g wet weight than amphibians (Nelson and Kruuk, 1997). It is possible, however, that VOCs from faeces and glands relate to foods eaten at different times because remains of some prey types can take up to 10 days to be passed by otters, although most take only 24 hours (Carss and Parkinson, 1996). Large variation in scent as a result of diet would have complicated or even prevented the detection of spraint scent differences relating to otter identity. In summary, however, diet appears unlikely to impact on scent, suggesting that there is no need to control for it in subsequent analyses.

3.5. Recommendations

3.5.1. Recommendations for developing methods

1. Systematic testing of SPME fibre type should be carried out (where possible on the same sample). Consideration should be given not only to the number of compounds recovered but also the type of compounds.

2. Test exposure time (a compromise may have to be made between maximising the number of components and completing work within a time frame that makes experimental work feasible).
3. Carry-over between samples should be tested by blank fibre analyses and, if necessary, extra reconditioning between sample analysis should be introduced.
4. The impact of sample storage conditions should be tested including the storage container if this varies between samples (which it did not in the current study).
5. Once fibre type, exposure time, and storage have been selected, the reproducibility of the chosen method should be tested by repeated sampling of several samples.
6. Some sort of automation for peak detection should be employed. Manual integration of each peak in very large datasets (such as that in Chapter 4) composed of a large number of samples, each with several hundred compounds, can be unfeasible and could take several years (Dixon et al., 2006). Custom libraries can be created in AMDIS by adding compounds found in a subset of samples. This can then be used to search other samples and therefore save time.

3.5.2. Method to sample and analyse VOCs from otter scent samples

Sample vials were placed in a water bath at 30°C to ensure a consistent temperature during sampling. Supelco Solid Phase Microextraction (SPME) fibres were used to collect VOCs eluting from samples, by exposing the fibre to the headspace of each sample for 45 minutes. The fibre used was StableFlex Divinylbenzene/Carboxen/PDMS (DVB/CAR/PDMS) 50/30μm bonded fibre. Fibres were conditioned according to manufacturer's recommendations and reconditioned for 10 minutes in a GC injection port at 260°C between each sample (or for 30 minutes if the fibre had not been used for several hours). An analysis of the fibre not exposed to any sample was conducted at least every sixth sample to detect any contamination or deterioration of the fibre, and fibres were replaced when damaged.

Following exposure, fibres were immediately analysed using GC-MS (Agilent 6890N/5973N). Fibres were injected manually and desorped for 2 minutes at 260°C in the split/splitless injection port fitted with an SPME liner (Supelco, Belefonte, PA, USA) in splitless mode. Samples were analysed on a 30 m, 0.25 mm I.D., 0.25 μm Zebron 5ms

capillary column (Phenomenex, Macclesfield UK) with helium as carrier gas at constant pressure (13.4 psi). The oven was held initially at 40°C for 2.5 minutes, then heated at 1.5°C min^{-1} to 100°C, followed by heating at 20°C min^{-1} to 300°C and held at 300°C for 4 minutes, resulting in a total programme time of 56.5 min. Temperature of transfer line, ion source and analyser of MS were set to 310°C, 230°C and 150°C, respectively. Mass spectra were recorded without solvent delay from m/z 35 to 550.

After every 4 to 6 samples 0.2µl of an external hydrocarbon standard (MA EPH Aliphatic Hydrocarbon Standard (Restek, Sauderton, UK) diluted 1:50 with n-hexane (Fisher Scientific AnalR)) was injected using an automatic liquid injector to check the performance of the GC-MS and for calculation of retention indices. This allows standardisation of retention times. Compounds were provisionally identified (minimum match factor between the deconvoluted component and the library spectra of 80%) and quantified using AMDIS (version 2.65) and the custom library or NIST Mass Spectral Library Version 2.0 (2005). Both mass spectral data and retention indices were used by AMDIS in identification. Peaks with a retention time below 2 minutes were not included in the analysis because signals with retention times close to the hold-up time of the system are not measurable with sufficient accuracy. The relative contribution of each peak to the overall scent profile was calculated i.e. data were normalised. Data were also standardised across compounds to ensure compounds were given equal weight in the analysis, irrespective of their size. Zero values were replaced with half the value of the lowest intensity compound measured to account for low values below the detection limit.

Chapter 4

Otter scent signals age, sex and reproductive status

This chapter was modified for publication:

Kean, E.F., Müller C.T. and Chadwick, E.A. (2011). Otter scent signals age, sex and reproductive status. Chemical Senses, 36, 555–564.

Author contributions: E.F.K designed and performed experiments, analysed data and prepared the manuscript. C.T.M provided analytical advice and commented on the manuscript. E.A.C conceived the study, collected samples and commented on the manuscript.

4.1. Introduction

Mustelids use scent marks for intra-specific communication and have been the subject of chemical research for over 140 years, probably as a result of the aggressively malodorous nature of their scent marks (Burger, 2005). Scent communication is common across social systems providing both a means for group cohesion for social species (Buesching et al., 2003) and a means of avoiding costly agonistic encounters with other individuals for solitary species (Erlinge et al., 1982). Hutchings and White (2000) propose two main functions of mustelid scent communication; communication of reproductive status and availability, and/or use of resources. Chemical analysis of scent marks can provide a useful insight into what information may be communicated about the identity of the signaller. Body condition and reproductive status are communicated through scent by badgers, *Meles meles* (Buesching et al., 2002b), and communication of individual identity has been found in a number of mustelid species (Zhang et al., 2003, 2005, Buesching et al., 2002b). Differences in VOCs relating to sex and/or age have been reported in other mustelid species' scent marks, for example, badgers (Buesching et al., 2002b), ferrets, *Mustela furo* (Zhang et al., 2005), Siberian weasel, *Mustela sibirica* and Steppe polecat, *Mustela eversmanni* (Zhang et al., 2002a). Although age and sex differences in mustelid scent secretions may seem ubiquitous they are not apparent in all species (Zhang et al., 2002b) or are only found at certain times of year (Service et al., 2001).

Early attempts to investigate *Lutra lutra* scent and the type of information communicated suggest differences between individuals (Gorman et al., 1978; Trowbridge, 1983) but very small sample sizes (n=2, n=3, respectively) limit conclusions. More recent attempts show no clear difference between male and female scent (Bradshaw et al., 2001) but the use of solvent extraction may have diluted some components of the scent below detection limits. To avoid this, a different extraction method is employed in the present study. Furthermore, previous studies focused comparisons only on the most abundant of components detected. This approach may have obscured differences between groups, as the compounds that produce the greatest discrimination between groups are not always the most abundant (Willse et al., 2005). Scents used in communication are complex mixtures and therefore ratios of multiple compounds can provide the necessary information where individual components do not (e.g. Pareja et al., 2009).

The elusive nature of the otter makes collecting spraints from known individuals in the wild impractical, and there are too few otters in captivity to investigate the chemical messages in spraint accurately. This research therefore makes use of otters found dead, which provide a unique opportunity to collect samples from individuals for which complementary parameters such as sex, age, reproductive status, size and indicators of health (e.g. parasite load and body condition index (Chadwick, 2007)) can also be recorded. As current survey methods (spraint surveys) assess distribution only (Crawford, 2010), and DNA analyses from spraints to identify individuals have a low success rate (Dallas et al., 2003), the potential to use chemical analysis of spraints for estimating the sex ratio and age structure of wild otter populations would offer new and affordable ways for non-invasive population monitoring. The aim of Chapter 4 is to discriminate between groups of otters (by age, sex and female reproductive status) based on profiles of volatile organic compounds from their anal scent gland secretions.

4.2. Materials and methods

Otter anal glands were collected according to the methods in Chapter 3 and samples were selected to represent different sexes, age groups and (for females) reproductive status (n=158, Table 4.1). Samples were stored for up to 5 years before analysis. The colour of the secretion was categorised by comparison to a colour chart, as light, medium or brown, mix of brown and white, or white. The smell of the secretion was categorised as faecal, sickly sweet, sweet,

old oil, other unpleasant, or other not unpleasant. The methods detailed in Chapter 3 were used to sample and analyse VOCs from the otter anal gland contents. Compounds in each sample were tentatively identified by comparison to the gland library created in the previous Chapter and detailed in Appendix 1. The identity of several compounds were confirmed by comparison to reference standards (supplied by Sigma-Aldrich) analysed under identical conditions. The main aim of this research was to identify differences between groups so positive identification of all components using reference standards was not necessary. Compounds found in less than five samples were considered unlikely to contribute to discrimination between groups because this is fewer than even the smallest group (pregnant females, n=9) and were removed.

Table 4.1. Number of otter *Lutra lutra* specimens used to investigate differences in volatile organic compounds (VOCs) from anal scent gland secretions.

	Reproductive status (females)	Adult	Sub-adult	Juvenile
Male	-	27	24	13
Female	Quiescent	25	25	10
	Pregnant	9	-	-
	Lactating	25	-	-

'Quiescent' refers to females that are sexually mature but not currently pregnant or lactating. Otters were assigned to an age-group (adult, sub-adult, and juvenile), based on body size and reproductive status (Chadwick 2007).

4.2.1. Statistical analysis

a) Univariate analysis

To examine differences in relative abundance of individual compounds between groups (sex, age-class and reproductive status, see Table 4.1) boxplots of all 432 compounds were visually examined and 20 compounds showing the greatest differentiation in data distribution between groups were tested statistically with a Mann-Whitney test. Although this introduces bias, it was considered the most appropriate approach because a multivariate test such as MANOVA would not be valid (data violate the assumption of having more dependent variables than cases in each cell), and individual statistical testing of each compound would incur a high risk of Type 1 error. To reduce bias, tests of the 20 compounds were conducted using Bonferroni adjusted alpha levels of 0.0025 per test.

b) Multivariate analysis

Principal Components Analysis (PCA) was applied to the full dataset (all compounds, following pre-treatment) to reduce the dimensionality of the data, using a correlation matrix to standardise across compounds. The resulting Principal Component scores (PCs) were used in discriminant function analysis (DFA). DFA was used to investigate whether variation in VOCs can be used to classify the scent samples by the age, sex or female reproductive status of the otter from which they were sourced. PC2 was log transformed in order to normalise the data, following addition of a constant (1.5) to make all scores >0. Prior probabilities were computed from group sizes. Subset validation was employed to validate the DFA; 70% of samples were used to create the model and 30% were used to test it. PCA and DFA were performed using SPSS version 16.0.

4.3. Results

Clear differences between samples were apparent to a human observer in both colour and odour of juvenile and adult samples. Adult samples tended to be darker than juvenile samples (64% of adult samples and 0% of juvenile samples were dark or medium brown, whereas 57% of juvenile samples and only 1% of adult samples were white). Sub-adult samples were intermediate between these extremes, with 42% dark or medium brown and 24% white (Figure 4.1a). Adult samples typically smelt like old oil or were sweet-smelling (76% of adults, 0% of juveniles), whereas juvenile samples typically smelt faecal (59% of juveniles, 3% of adults). Again, sub-adult samples were intermediate, with 37% old oil or sweet and 22% faecal (Figure 4.1b).

Complexity of chemical profiles varied considerably; the number of compounds per sample ranged from 36 to 165 (mean 112 ± 25.34) (see Figure 4.2 for chromatogram examples). There was no significant difference between the eight groups in profile complexity (i.e. the number of compounds detected per sample ($\chi 2=10.536$, $p=0.160$, Kruskal Wallis) (Figure 4.3)). Across all samples a total of 432 compounds were found of which 268 were provisionally identified using NIST library data. These comprised a complex mixture of small organic acids (C3–C6), their esters, alkanes (C7–C16), alkanols (C5–C8), aldehydes and ketones (C4–C12), aromatic compounds, furanes and nitrogen, and sulfur-containing compounds, for example, pyrazines, thiols, and sulphides (listed in Appendix 1). Typically

the largest peak, and common to all samples, was identified as benzaldehyde (identity confirmed by comparison to reference standard).

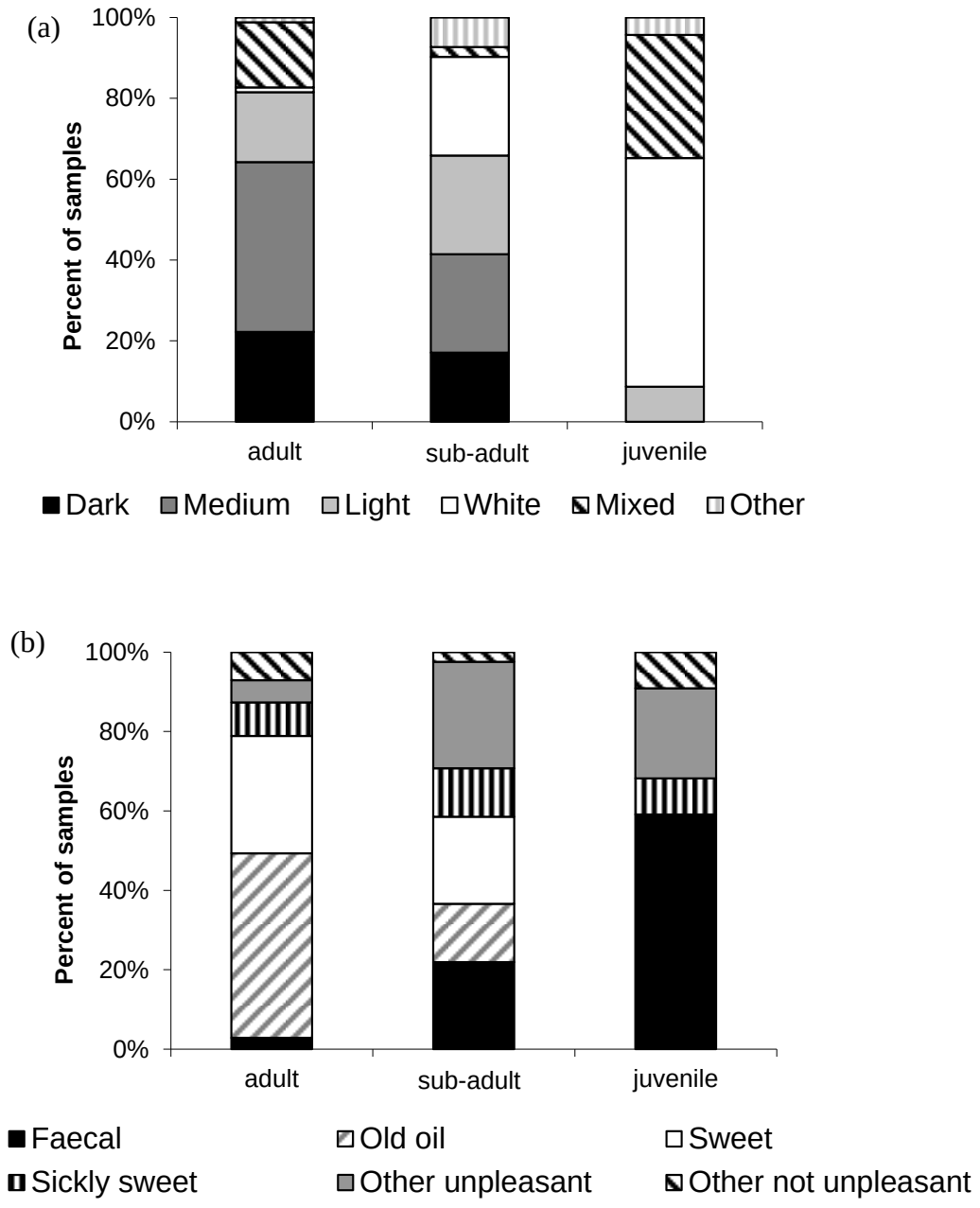


Figure 4.1. Human perception of (a) colour and (b) scent of material expressed from otter anal scent glands.

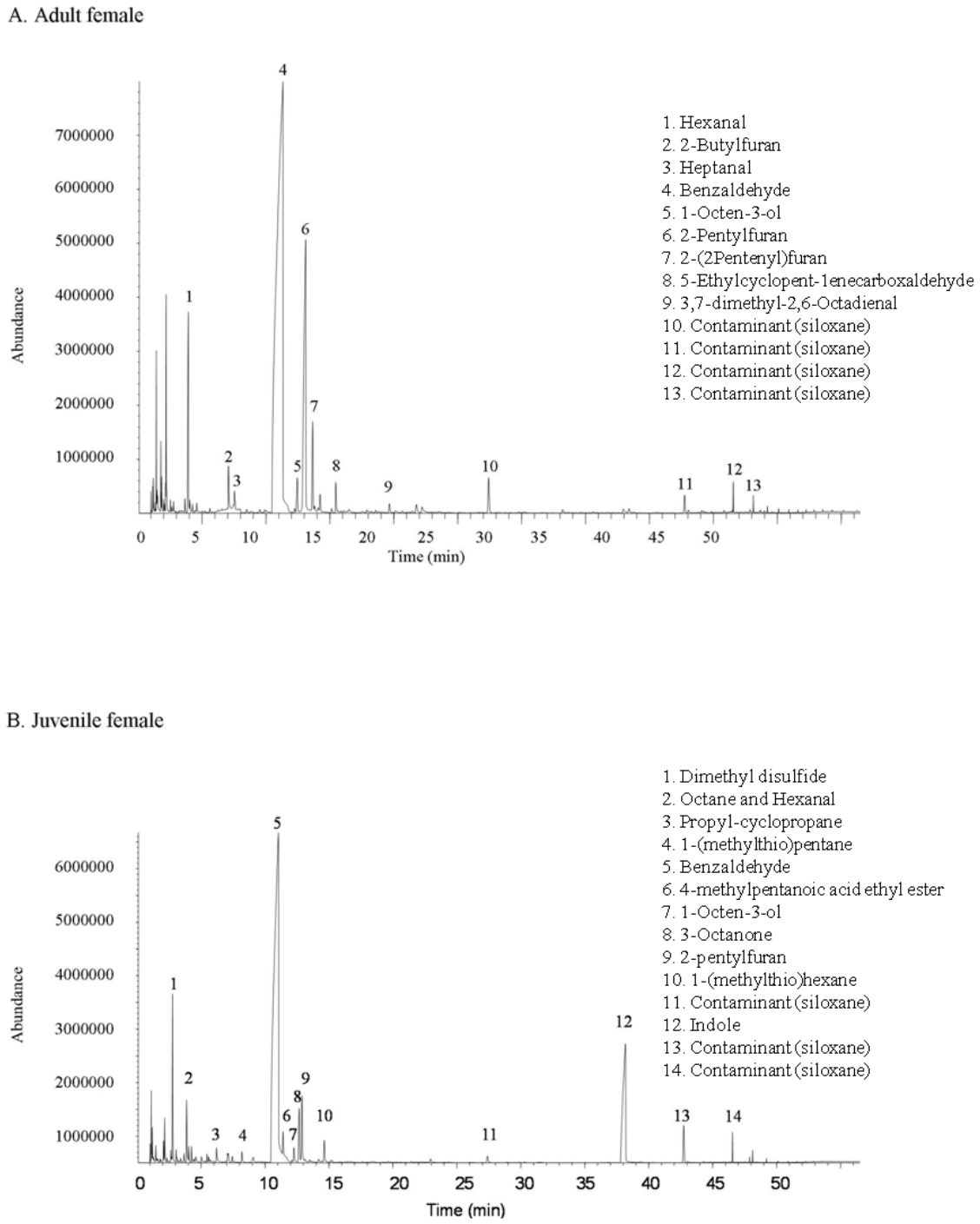


Figure 4.2. Example TICs (total ion chromatograms) from GC–MS analysis of VOCs from anal gland contents of an adult female (A) and a juvenile female (B) otter. Provisional identifications of the largest peaks are given, however, there are many more compounds at lower abundances.

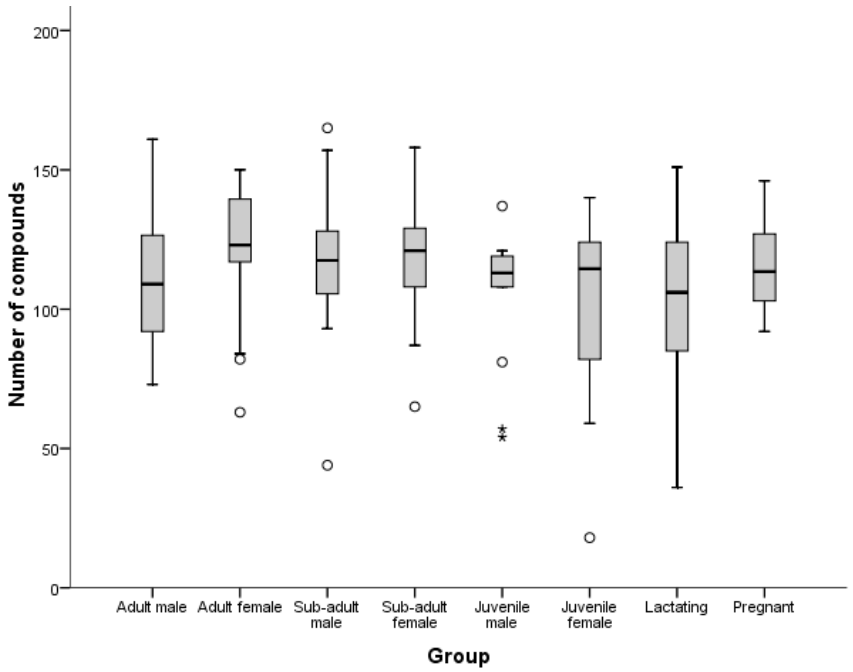


Figure 4.3. Number of volatile organic compounds detected in samples from eight groups of otter based on age, sex, and reproductive status.

4.3.1. Discrimination between groups

a) Univariate analyses

Single components provided some discrimination between age groups but did not enable discrimination by sex or reproductive status. Two peaks differed significantly between adults and juveniles, which were identified (by comparison to reference standards) as indole (mean adults =0.00 ± 0.01, mean juveniles =0.11 ± 0.06; z=-6.876, p<0.001; Mann-Whitney U Test) and 2-pentylfuran (mean adults =0.15 ± 0.10, mean juveniles = 0.01 ± 0.16; z=-6.614, p<0.001; Mann-Whitney U Test).

b) Multivariate analyses

Seven Principal Components (PCs), explaining 30% of the total variance of the 432 compounds found, were used in further analysis. DFA using all seven PCs correctly cross-classified only 39% of samples to the eight groups; adult male and female (quiescent), sub-adult male and female, juvenile male and female, pregnant female and lactating female (Table 4.2.). Within this, however, no pregnant or lactating females were ever misclassified as male or juvenile. PC 1 scores alone clearly separate pregnant and lactating females from juveniles (Figure 4.4).

Table 4.2. Results of DFA subset validation of otter anal scent gland contents.

	% of samples correctly classified	Discriminant function	Wilk's λ	χ^2	p
Eight groups	39.0 (no pregnant or lactating as male or juvenile)	1	0.201	170.940	<0.001***
		2	0.604	53.783	0.029*
		3	0.740	32.086	0.156
		4	0.855	16.684	0.406
		5	0.927	8.021	0.532
		6	0.988	1.244	0.871
		7	0.999	0.055	0.814
Three Age groups	61.0 (no adults as juveniles and 1 juvenile as adult)	1	0.417	95.236	<0.001***
		2	0.917	9.483	0.148
Sex	65.9 (female 74.1%, male 50%)	1	0.715	36.678	<0.001***
Adult male and female (excluding pregnant and lactating females)	73.7 (female 78%, male 70%)	1	0.572	14.781	0.039*
Sub-adult male and female	61.1	1	0.581	13.826	0.054
Juvenile male and female	100	1	0.465	9.565	0.215

The model is created using 70% of samples and 30% are tested with it. Wilk's λ is a measure of how well each function separates groups. Smaller values indicate a greater discriminatory power of the function. The associated chi-squared value tests the hypothesis that the means of the functions are equal across the groups. Significant values indicate that the discriminant function does better than chance at separating the groups. Significant discriminant functions: *p < 0.05, **p < 0.01, ***p < 0.001.

DFA of just the age groups correctly cross-classified 61% of samples but no adults from the subset were misclassified as juveniles, and only one juvenile was misclassified as an adult. Sub-adult samples were often misclassified as adult or juvenile (Table 4.2.). Overall, 66% of samples were correctly cross-classified to their sex group, with females (74%) much better classified than males (50%). When adult data were analysed separately, and pregnant and lactating females were excluded, sex classification was better, with 70% of males and 78% of females correctly cross classified. Juvenile sex classification was also improved by analysis within age-group, but this was not significant and the sample size for subset validation (n=4) was very low.

No significant differences were found between correctly and incorrectly classified samples in sample weight, number of compounds in each sample or slight methodological variations (e.g. different vials used for collection). This suggests the method is robust to slight variations.

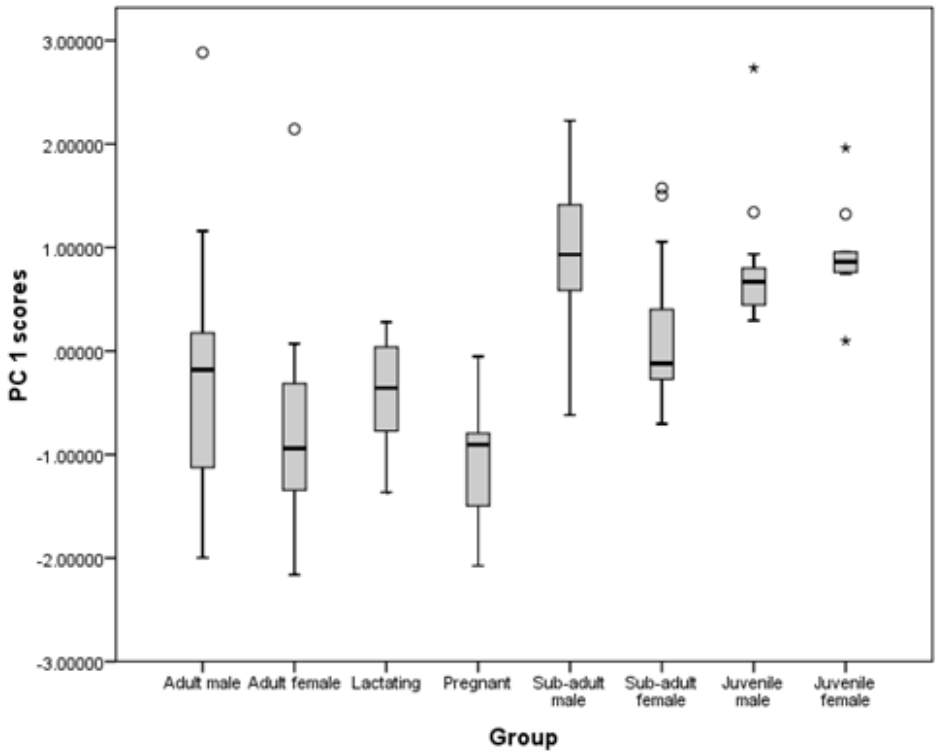


Figure 4.4. Difference in PC1 value from chemical profile of anal gland secretion between the 8 different otter groups investigated. Statistical discrimination was performed using 7 PCs (test statistics are presented in Table 4.2), here only PC1 is plotted for illustrative purposes.

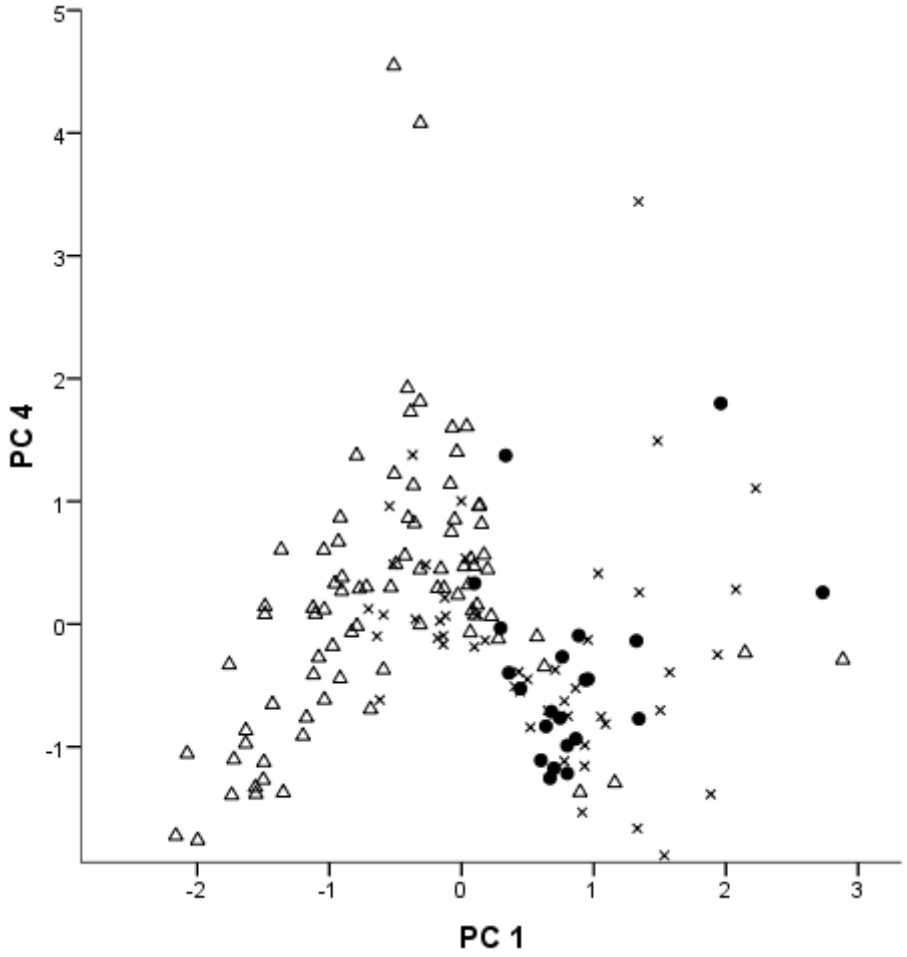


Figure 4.5. PC1 and PC4 from chemical profiles of otter anal gland secretion showing some differentiation between age-groups (Δ adult, x sub-adult and • juvenile). Statistical discrimination was performed using seven PCs (test statistics are presented in Table 4.2), PC1 and PC4 are plotted here as they were most important in the DFA.

Two PCs were commonly important in the discrimination of age group, sex and adult sex. PC1 and PC4 were the discriminating variables with the highest pooled within-groups correlations with the first discriminant function for age group (PC1= -0.728, PC4 =0.251 tied with PC 2=0.251). sex (PC1=0.592, PC4=-0.643, next highest PC5 =0.158) and adult sex DFAs (PC1=0.375, PC4=-0.311, next highest PC3=0.222). Several furans loaded heavily onto PC1 and isomers of 2-Octene loaded heavily onto PC4 (see Table 4.3 for compounds that load heavily on PC1 and PC4). PC3 is made up almost entirely of butanoic acid esters. The largest peak that loaded heavily on PC1 was 2-pentylfuran, which alone allows significant differentiation between adults and juveniles.

Table 4.3. PCA loadings (with values greater than +/- 0.7) of PC1 and PC4 from volatile analysis of *Lutra lutra* anal gland secretions.

	Retention Index	Occurrence (number of samples)	Mean relative abundance (% area) ± standard deviation
PC1			
2-Propylfuran	834	48	0.30±0.17
Hexanal*	837	141	4.81±4.78
Unknown 87	838	89	0.04±0.04
2 Butylfuran	890	124	0.61±0.61
Heptanal*	898	128	0.26±0.26
Unknown 85	914	90	0.07±0.06
Unknown 78	920	42	0.06±0.05
2-Pentylfuran*	986	156	10.25±9.61
3-Octanone*	991	145	1.86±2.89
2-(2-Pentenyl)furan	997	110	2.24±2.00
Octanal*	1000	44	0.14±0.09
3-Pentylfuran	1003	66	0.04±0.02
5-Ethylcyclopent-1-enecarboxaldehyde	1020	114	0.85±0.88
Unknown 86	1076	77	0.04±0.03
2-Hexylfuran	1082	64	0.07±0.05
Potential triene C13	1314	72	0.22±0.14
Potential diene C13	1319	61	0.06±0.04
PC4			
(E)-2-Octene*	840	157	0.66±0.75
(Z)-2-Octene	844	125	0.63±0.56
Unknown 66	847	149	0.17±0.17
Potential C11H18 isomer	1070	44	0.25±0.20
Cyclooctanemethanol	1073	118	0.07±0.07
Potential C11H18	1078	121	0.23±0.24
2,9-Undecadiene	1102	140	0.23±0.25

*Compound identity confirmed by comparison to reference standards.

4.4. Discussion

The colour and human perception of the smell of anal gland secretion differed between adult and juvenile otters, but not between the sexes. The lack of colour differentiation between otter sexes differs from observations recorded for other species such as beavers (Schulte et al., 1995) and badgers (Buesching et al., 2002a), where clear colour differences in scent gland secretions are recorded. Several of the compounds involved in discrimination have been found in other mustelid scent secretions; 2-pentylfuran and 3-octanone in *Gulo gulo* (Wood et al., 2005), and both hexanal and heptanal in *Mustela furo* (Zhang et al., 2005). Indole is seemingly ubiquitous in the scent secretions of mustelid species; *Mustela putorius*, *M. nivalis*, *M. erminea* (Brinck et al., 1983), *M. vison* (Brinck et al., 1978; Zhang et al., 2002b)

M. furo (Crump, 1980a; Brinck et al., 1983; Zhang et al., 2005), *M. eversmanni, M. sibirica* (Zhang et al., 2003), and Wolverine, *Gulo gulo* (Wood et al., 2005). Indole and benzaldehyde (the largest compound in most samples and also found in several other mustelid species (Wood et al., 2005; Zhang et al., 2005; Brinck et al., 1983)), were previously thought to be absent from otter scent (Brinck et al., 1983), exemplifying how employment of different analytical methods can improve results. Isomers of 2-octene are previously unreported in mustelid scent secretions, although they do occur in human faeces (Garner et al., 2007) and were found here to load heavily on PC4, important in discrimination between groups. Although the methods used here reveal several compounds 'new' to mustelid or otter scent, it is recognised that these methods may also have limitations and not sample all VOCs from otter scent.

4.4.1. Age differences

Adult and juvenile otter anal gland VOCs differ in the relative abundance of indole and 2-pentylfuran; more complex multivariate differences also occur (as demonstrated with PCA and DFA) even though only one of these compounds (2-pentylfuran) loaded heavily onto the PCs used in DFA. Although only 61% of samples were classified correctly to their age group following subset classification, there was only one misclassification between adults and juveniles. Age is, of course, continuous, and there is currently no accurate method for ageing otters (Sherrard-Smith and Chadwick, 2010). Otters used in this study were assigned to the given age groups (adult, sub-adult and juvenile) at post-mortem, based on body size and reproductive status (Chadwick, 2007), which because of differences in development rates, may not always accurately reflect actual age, leading to unavoidable overlap between categories. Although there is some misclassification of scent profiles between adults and sub-adults, as well as between juveniles and sub-adults, we can successfully discriminate between adults and juveniles. This difference in development stage would be of most interest for scent discrimination, rather than chronological age. In a review of mammalian social odours, Brown (1979) found little chemical evidence for age specific odours. Since then, however, age differences in chemical composition of scent have been found in some species (Buesching et al., 2002b; Osada et al., 2003; Schaefer et al., 2010). In the current study, differences in scent between juvenile and adult anal gland samples were apparent even to the human nose; juvenile samples were much stronger and faecal smelling. Indole, which was present in all juvenile samples (usually at high relative abundance) but found in very few

adults, does have an unpleasant odour at high concentrations (Lewis, 2007). Adult otter anal gland samples were more frequently sweet smelling and spraint is commonly described by field workers as smelling of jasmine or freshly cut hay.

The spraints of otter cubs are usually larger than adult spraints, and adult male spraints are usually smaller than those of females (Kruuk, 2006). This could be because adult males deposit spraint more frequently for communication purposes whereas cubs simply spraint for faecal elimination. The distinct difference in scent of juveniles may simply be an immature body function or a result of dietary differences, rather than functional signalling, as it is likely that many of the otters categorised as juveniles in the present study were not fully weaned (based on morphometric data 'juveniles' were thought to be <20 weeks; otters are weaned at approx 14 weeks (Harris and Yalden, 2008)). There is no firm evidence of the age at which otter cubs deposit spraint as a marking behaviour rather than for faecal elimination. Scent marking is characterised by repeated deposition of small amounts of material at selected sites (Kleiman, 1966), whereas deposition purely for faecal elimination tends to be more voluminous and have no pattern of deposition. Erlinge (1968) mentioned two captive cubs displaying marking behaviour when they were first observed at around 8-9 months, but observations were not made prior to this and so this behaviour may have developed at a younger age. Other observations of juvenile otter sprainting behaviour have been made at around 5 months of age (Green, 2000; Polotti et al., 1995) so the otters used in the present study may not have been scent marking.

4.4.2. Sex differences

Sex differences in VOCs were found between adult male and female otters but not in younger otters, suggesting a role in sex specific communication, such as mate attraction. The nature of sex differences in scent vary between species. Differences in the relative abundance (analog coding) and presence/absence (digital coding) of individual compounds have both been found in mustelids (Zhang et al., 2003, 2005). Multivariate analyses combine analog and digital coding, and have been used to reveal subtle signalling differences between organisms involving complex mixtures of compounds (e.g. results presented in this chapter and in humans (Penn et al., 2007)).

Despite chemical communication of sex, for the purpose of mate attraction or choice, being omnipresent in the vast majority of living organisms (Johansson and Jones, 2007), an absence of sex differences in scent marks has been found in some mustelids, for example mink, *Mustela vison* (Brinck et al., 1978; Zhang et al., 2002b). The apparent disparity between species in communication of sex through scent marks may reflect differences in ecology or limitations in the techniques used to analyse the scent marks either statistically (as described above) or chemically (for example the extraction method used, or detection limits of GCMS). A larger sample size combined with different methods of chemical and statistical analysis in our study allowed us to build on previous findings by Bradshaw et al. (2001) and to discriminate between adult male and female otters. Similarly, the use of different sampling techniques improved VOC analysis from two other mustelid species (Zhang et al., 2003). Analysis of different scent secretions from the same species may also reveal differences that were previously thought to be absent, for example sex differences were found in badger subcaudal (Buesching et al., 2002b) but not anal gland secretions (Davies et al., 1988). Otters do not have subcaudal glands but it is possible that some messages are communicated in non-volatile components of otter scent, as is well documented in mice (Hurst et al., 2001; Nevison et al., 2003); analysis of non-volatiles may reveal simpler differences between otter sexes.

4.4.3. Female reproductive status

The clearest differences between the eight groups of otters using DFA was between pregnant or lactating females and male or juvenile otters. Dietary or hormonal differences may explain the differences between pregnant or lactating females and juvenile otters. Reproductive status also affects the scent of female badgers (Buesching et al., 2002b). Parental care and therefore the recognition of young is an important factor in the evolution of social odours in mammals and partly explains scent differences between individuals (Brennan and Kendrick, 2006). Generalised scent signals of pregnancy or lactation also exist, for example nipple-search pheromone in humans and other mammals (Porter and Winberg, 1999) or the generalised attraction to nest odours in gerbils (Gerling and Yahr, 1982). Female otters are said to be very secretive when they are pregnant or lactating and natal are dens hard to find (Kruuk, 2006). Signalling their reproductive status to males is likely to be disadvantageous, as males are known to commit infanticide (Simpson and Coxon, 2000). Pregnant females or those with very young cubs are said to spraint in water (Jenkins and Burrows, 1980; Kruuk, 2006), and this might help to hide the scent differences shown in the current study.

In summary: these results indicate simple age differences in the VOCs from anal scent gland secretion of Eurasian otter *Lutra lutra,* and more complex multivariate differences with sex and reproductive status of adult otters. These results are a first step towards further understanding of otter scent communication, and provide evidence of categorical differences which might be used in the future to help monitor age and sex ratios of wild populations. The complexity of sex differences may make this difficult.

Chapter 5
Individual scent fingerprint and variation with the female reproductive cycle

5.1. Introduction

The traits of signalling identity and the ability to recognise individual conspecifics have evolved because of the associated fitness benefits for both signaller and receiver (Tibbetts and Dale, 2007). For example, individual recognition may allow individuals to invest resources and care only in their own offspring (reviewed by Lévy et al., 2004), to avoid potentially costly interactions with stronger opponents (Gosling, 1982), or to aid the identification of mates and avoid inbreeding (Hurst, 2009). Some of these behaviours, however, may be possible without individual identification (e.g. inbreeding may be avoided by kin/non-kin cues rather than individual identity). In mammals individual identity may be communicated through vocal or visual cues but scent is the most common modality (Brown and Macdonald, 1985; Wyatt, 2003; Thom and Hurst, 2004; Brennan and Kendrick, 2006). Thom and Hurst (2004) identified three key characteristics of individual scent cues; independence from other background variation, a high degree of diversity, and temporal stability. Variation in histocompatibility complex (MHC) is commonly thought to be the mechanism for individually distinct scent cues, but this was recently disproved in mice which called into question the assumption of a vertebrate-wide mechanism (Cheetham et al., 2007).

There are two methods commonly applied to the study of individual recognition; bioassay and chemical analysis. Both were criticised by Halpin (1986) over 25 years ago; chemical analysis because it is an indirect measure of individual recognition, and bioassays because they frequently test familiarity of scents rather than true individual recognition. Although improvements have been made, Thom and Hurst (2004) more recently reviewed the field and found many of Halpin's concerns had not been addressed, and in particular more focus is needed on function. Despite the criticism of chemical analysis, it is the most appropriate method in this chapter, as the primary focus was to investigate individual differences in otter scent for the purpose of

developing a monitoring technique; indication of communication function is potentially a secondary outcome. Additionally, for species that are difficult to manipulate in captivity (for practical or legislative reasons), chemical analysis of scent marks offers a more viable option than bioassay.

Since the early investigations into individual scent (e.g. black tailed deer; Müller-Schwarze, 1971), scent signatures have been investigated in many species. Methods of early investigations were simplistic, for example presentation of chromatograms, compared to the complexity of analytical and statistical methods available today. Chemical evidence of individually distinct volatile scent signatures now exists for many mammalian orders: ungulates (Müller-Schwarze, 1971), rodents (Sun and Müller-Schwarze, 1998b), carnivores (Hagey and Macdonald, 2003; Burgener et al., 2009), primates (Scordato et al., 2007; Smith et al., 2001; Setchell et al., 2010), bats (Safi and Kerth, 2003) and lagomorphs (Goodrich and Mykytowycz, 1972). Differences between individuals' scent exist even for species such as ourselves, for whom scent communication is assumed to be unimportant (Penn et al., 2007) and chemical human scent barcoding was recently suggested as a forensic tool (Curran et al., 2010).

Although Thom and Hurst (2004) identified temporal stability as a key characteristic of an individual identity cue, empirical evidence suggests this is not always the case. Individual patterns in scent are stable for adult mink but not juveniles (Brinck et al., 1978). There can be seasonal differences in scent (Scordato et al., 2007; Stoddart et al., 1975) and individual signatures are stronger during the breeding season for *Meles meles* (Buesching et al., 2002c). This suggests that individual scent identity may be influenced by reproductive hormones.

There is a variety of evidence that indicates scent gland secretions are related to hormones. Sex and age differences in scent alone indicate hormonal control (Ma et al., 1999). Changes in odours following neutering and hormone supplementation have provided evidence of a link between endocrine status and scent in house mice (Schwende, et al., 1986) and wolves (Raymer et al., 1986). The use of pheromone (Z)-

7-dodecen-1-yl acetate by female elephants to communicate oestrous stage is well-documented (Rasmussen et al., 1996, 1997). Flehmen is a well-known male behavioural response in elephants that enabled the reliable use of bioassay to identify the female sex pheromone (Rasmussen et al., 1997). Studies of various mammals provide behavioural evidence for chemical communication of oestrous state; examples include baboons (Clarke et al., 2009), giant pandas (Swaisgood et al., 2002) and hamsters (del Barco-Trillo, 2009) but few have combined chemical analysis of scent marks with more robust measures of oestrous state. Exceptions include studies where oestrous stage is ascertained by rectal examination of reproductive organs, only possible in domesticated animals such as horses (Ma and Klemm, 1997), cows (Kumar et al., 2000) and buffaloes (Rajanarayanan and Archunanand, 2011). In these studies, specific VOCs were found in urine only during oestrous and not in other stages of the cycle. Microscopy of mouse vaginal smears to identify oestrous stage has been coupled with scent analysis and revealed concentrations of 28 urine volatiles, 10 of which increased and one declined during oestrous (Andreolini et al., 1987).

Within the mustelid family, individual scent signatures have been reported, by visual examination of chromatograms, in *Mustela ermine* (Erlinge et al., 1982), *M. vison* (Brinck et al., 1978) and *Meles meles* (Buesching et al., 2002b), or by examination of individual chromatogram peaks in *Mustela evermanni* and *M. sibirica* (Zhang et al., 2003). Stronger evidence comes from multivariate statistical analysis of scent marks from *Meles meles* (Buesching et al., 2002c) and *Mustela furo* (Zhang et al., 2005). As reported previously (Chapter 1) , there has been little systematic observation of any otter species or chemical analysis of their scent marks to determine the function of sprainting behaviour. Early attempts to examine individual differences in *Lutra lutra* scent suggest individual differences (Gorman et al., 1978; Trowbridge et al., 1983) but small sample sizes (n=2 and n=3) limit conclusions. Additionally Gorman et al. (1978) did not conduct any statistical analysis and Trowbridge (1983) conducted multiple univariate analyses, potentially introducing Type 1 errors. A low number of repeat samples from known individuals in captivity prevented the assessment of individual scent signatures of Eurasian otters by Davies (2009).

Significant wild population declines led to extensive interest in breeding Eurasian otters in captivity in the 1980s and 1990s. Information on oestrous cycles, however, remains sparse. Female otters are said to be polyoestrous (Broekhuizen et al., 2007) and evidence to support this comes from post-mortem studies that have revealed that otters can breed at any time of year (Chadwick and Sherrard-Smith, 2010: Hauer, 2002: Heggberget and Christensen, 1994). It has been suggested that within periods of oestrous, female otters may be induced ovulators (Broekhuizen, 2007; Jorga et al., 2004). Observations of behaviours associated with oestrous (rolling and moaning, copulation) of one captive female indicated an oestrous duration of 11-14 days with an interval of 30-40 days (Gorman et al., 1978). Faecal hormone analysis has been used successfully to describe the oestrous cycles of sea otters (*Enhydra lutris*) (Larson et al., 2003), North American river otters (*Lontra Canadensis*) and Asian small-clawed otters (*Amblonyx cinereus*) (Bateman et al., 2008), but there are no published studies for the Eurasian otter (although there are data from the urine of a single female otter in Trowbridge, (1983)). Faecal steroid analysis has been used to assess sex, age and reproductive status in wild populations of *Lutra lutra* (Tschirch et al., 1996; Kalz et al., 2006) with methods validated on captive otters. The progesterone levels associated with pregnancy (>200 or 220ng g^{-1}) given by Tschirch et al. (1996) and subsequently adopted by Kalz et al. (2006), were based on 25 samples from one adult female and an unspecified number of pregnant females in zoos. This limited quantification of the variation in progesterone levels within individuals makes their assumptions of levels indicative of pregnancy questionable.

It is widely presumed that male otters use scent to detect a female in oestrous (Chanin, 1985; Laidler, 1982; Kruuk, 2006) although this has never been tested. Lack of detailed records of female otter oestrous behaviour or male otter responses to female scent prevents the use of bioassay and physical examination of reproductive organs is too invasive for a wild animal in captivity. The relatively new technique of non-invasive sampling of faecal steroid hormones is more suitable to assess oestrous stage of wildlife. Sampling faecal steroid hormones also avoids ethical concerns associated with blood sampling and the potential impacts that stress resulting from handling may have on results (Schwarzenberger, 2007).

The first part of this chapter examines variation in the scent of otter spraints between individuals. The second part examines female reproductive cycle in the Eurasian otter and tests whether this is associated with variation in spraints scent. Because scent gland samples collected from otter carcasses represent a single time point, it is not possible to use samples taken at post-mortem as in the previous chapter. Instead it was necessary to analyse scent samples collected repeatedly from the same individual, using animals in captivity. This allows a number of aims to be addressed: (1) describe the otter oestrous cycle, (2) ascertain whether the stage of the oestrous cycle is signalled by changes in scent, and (3) assess how much within-individual variation occurs in female otter scent over a three month period. The latter is one of several important steps in the current studies wider aims (the development of scent profiling as a method to enable identification of individuals) because without quantifying how variable signals are within individuals it will not be possible to determine whether scent can be used to discriminate between individuals.

5.2. Methods

5.2.1 Individual identity

Spraints were collected from 17 otters in captivity from six different centres in the UK, Germany and Spain. Some otters were housed individually ensuring the origin of samples collected, for those who were housed in pairs spraints were collected only when the otter that deposited the spraint could be observed. Between one and five samples were collected from each otter, with a minimum interval of one day and maximum interval of 354 days between samples (Table 5.1). Samples were collected and stored in sterile plastic tubes or plastic zip lock bags, and stored at -20^{o}C for a maximum of 19 months before analysis. Samples were defrosted, transferred to glass vials and analysed using the methods detailed in Chapter 3.

Table 5.1. Spraints collected from captive Eurasian otters to investigate individuality in spraint odour by analysis of volatile organic compounds (VOCs) using solid phase microextraction (SPME) and gas chromatography mass spectrometry (GCMS).

Location	Otter	Age (years)	Sex	Sampling period (days)	Sample n
RSPCA Eastwinch, UK	19161	<1	Male	9	5
RSPCA Westhatch, UK	16996	<1	?	1	1
British Wildlife Centre, UK	Minnie	12	Female	115	3
	Lilly	3	Female	1	2
	Oscar	12	Male	10	2
	Stirling	11	Male	18	2
	Thistle	7	Female	112	2
Newforest Wildlife, UK	Alpha	17	Male	354	2
	Sirius	1	Male	129	3
	Grace	2	Female	129	2
Otter Zentrum, Germany	Desiree	4	Female	68	3
	Evi	<1	Female	68	3
	Henri	2	Male	62	3
	Naima	7	Female	38	3
	Teufel	5	Male	64	3
Terra Natura Murcia, Spain	Vagui	4	Female	8	4
	Cuca	3	Female	12	4

5.2.2 Female reproductive cycle

a) Sample collection

Spraint samples were collected from six adult female otters each housed alone at Krakow Zoo, Poland; Miejski Ogrod Zoologiczny, Poland; Terra Natura, Spain; Fundación Zoo de Santillana, Spain. Spraint samples were collected daily from each otter within 24 hours of deposition, for a minimum of one month between April and July 2009. Disposable gloves were used to collect samples to prevent contamination. Samples were placed into aseptic plastic tubes and stored at -20^{o}C until the end of the sample collection period. Samples were shipped on dry ice to the UK and then stored at -20^{o}C until analysis. Samples from alternate days were selected for analysis of reproductive steroid metabolites and the remaining samples were used for scent analysis of VOCs.

b) Reproductive hormone analysis

Analyses for progesterone metabolites using enzyme-linked immunosorbent assay (ELISA) were conducted by E. Bujak, Zoological Society of London (ZSL). A selection of samples that were most likely to contain oestrogen (due to low progesterone levels) were analysed using ultra-performance liquid chromatography (UPLC) to identify oestrogen metabolites. No oestrogen metabolites were detected so no further analysis was conducted for oestrogen metabolites.

Three extraction methods were compared for a sub-set of three faecal samples; using either 100% methanol, 80% methanol/distilled water or potassium hydroxide (KOH)/petroleum ether. The method using 100% methanol was selected for extraction of faecal samples as this method resulted in optimum extraction of hormone metabolites. After thawing, samples were dried in an oven (Gallenkamp I incubator; Weiss Gallenkamp, UK), at 60^{o}C, for 18 hours. The dried faecal material was transferred to zip-lock bags and pulverised by hand to produce a homogenous, finely granulated powder. Then, 0.1g of each sample was weighed and placed into a 5ml glass test tube and 1ml analytical grade methanol was added to each tube. Samples were vortexed for 10 minutes, followed by centrifugation (Coolspin; MSE-Fisons, UK) at 2400 rpm, at 4^{o}C, for 20 minutes. The methanolic supernatant was removed using fresh Pasteur pipettes and transferred into 2ml vials and stored at -20^{o}C until assayed. Sample analysis was conducted within one month of extraction.

Progestagen assays were performed in microtiter plates, pre-coated with goat antimouse IgG (Sigma M8890; Sigma Chemical Co., Poole, UK) and stored at 4^{o}C in an airtight container until use. Before use, plates were washed with washing solution (distilled water containing 0.05ml l^{-1} tween 20). Sample extracts were diluted 1:8 in assay buffer (2.42g trishydroxyaminomethane; 17.25g sodium chloride; 1.0g bovine serumalbumin; 1.0ml tween 80; 1.0l distilled water, pH 7.5 with 1mol hydrochloric acid). Progesterone (Sigma P0130; Sigma Chemical Co., Poole, UK) was diluted in assay buffer to obtain concentrations of 0.07, 0.15, 0.3125, 0.625, 1.25, 2.5 and 5ng m^{-1}, to provide a standard curve for the assay. 50µl standards, controls (high- (~ 70%) and low- (~ 30%) binding internal controls) and samples were added to the wells, in

duplicate. This was followed by the addition of 100µl biotinylated progesterone 1/750000 (EL57; Dr. E. Möstl, Veterinary Medicine University, Vienna, Austria) and 100µl primary antibody 1/200000 (CL425 monoclonal mouse antibody; Quidel Corporation, San Diego, USA; Dr. C. Munro, University of California Davis, California, USA).The antibody cross-reacts with a large number of progesterone metabolites (Graham et al., 2001). The plate was incubated overnight at 4^{o}C. Afterwards, the plate was washed four times and 250 µl streptavidin conjugated to horseradish peroxidase 1/750 (Sigma S5512; Sigma Chemical Co., Poole, UK) was added. The plate was incubated at 4^{o}C for one hour before washing four times. 250µl substrate solution (75ml of substrate buffer; 21.01g citrate buffer, 1.0ml tween 80 and 1 litre distilled water), 30µl hydrogen peroxide and 1 x 30mg ophenylenediamine dihydrochloride tablet (Sigma P8412; Sigma Chemical Co., Poole, UK) was added. The plate was incubated at room temperature, under a light-proof box, for 20 minutes, before the enzymatic reaction was stopped with the addition of 50µl stopping solution (670ml distilled water and 330mL 95-97% concentration sulphuric acid). Absorbance of the plate was read immediately, at 490nm (reference 650nm) on a microplate reader (EMax Precision Microplate Reader; Molecular Devices, MDS Inc., Sunnyvale, CA, USA) and Progesterone concentration was expressed on a dry-faecal-weight basis.

c) Sampling and analysis of volatile organic compounds (VOCs)

Sampling and analysis of VOCs was conducted at the NERC Life Sciences Mass Spectrometry Facility, Bristol University. Samples were defrosted in a refrigerator overnight and transferred from plastic tubes into Solid Phase Micro-extraction (SPME) vials prior to analysis. VOCs were sampled from the headspace above samples using SPME following the methods developed in Chapter 3. At regular intervals (at least once every six samples), fibres were reconditioned at 260°C for 30 minutes and blank analyses were run. This was done by exposing the fibre to an empty SPME vial under the same conditions used for samples and analysed in the same way. This allowed detection of contamination or deterioration of the fibre, and also enabled the identification of peaks originating from vials or fibres rather than samples.

VOCs were analysed by Gas-Chromatography Mass-Spectrometry (GC-MS). Following exposure, fibres were immediately injected manually (to a depth of 3.5; testing was done to ascertain this depth) into to the inlet of a ThermoQuest Trace MS with a HP-1 50m, 0.32 mm I.D., or a ZB-1 60m, 0.32 mm I.D. column. The GC-MS inlet was fitted with a PTV injection port and fibres were desorped in splitless mode at 260°C for 10 minutes. The GC oven was initially held at 40°C for 2.5 min, heated by 1.5°C min^{-1} to 100°C, then 20°C min^{-1} to 300°C and finally held at 300°C for 4 min, resulting in a total programme time of 56.5 min. Mass spectra were recorded from m/z 50 to 650. The performance of the GC was monitored with a 1.0µl liquid injection of five fatty acids (FAME mix). Compounds were provisionally identified by comparison to the NIST library using Xcalibur (2.0.6) software (Thermo Electron Corporation).

5.2.3. Data analysis

To examine individual identity of spraint scent a new AMDIS library was created using a subset of 22 spraint samples collected from captive otters. This was refined by removing rare compounds: those found in less than three samples. All peaks were tentatively identified in AMDIS by comparison to this new custom library. Pre-treatment of data followed the methods in Chapter 3 (i.e. normalised, standardised and zeros replaced with half minimum).

In the female reproductive cycle experiment, VOCs that were found in blank samples were excluded and data were normalised, standardised and zeros replaced as above. As spraint was collected daily but analysis for progesterone and VOCs were conducted on samples from alternate days, for statistical analysis VOC data were made parallel with the progesterone level from the preceding day (i.e. Day 1 progesterone level was combined with Day 2 VOCs, Day 3 progesterone level was combined with Day 4 VOCs and so on). Although there is a time lag between hormone production and excretion in faeces that is variable between and within species (Kleiman et al., 2010), the temporal relationship between internal state and scent excretion is unknown. This is an area for future research.

Permutational Multivariate Analysis of Variance (PerMANOVA) was used to investigate which variables explained variation in the scent profile (all VOC abundaces), where the explanatory variables were otter identity, otter location and progesterone level. For the individual identity experiment this tested the null hypothesis that there is no difference between individual otters in the scent of their spraint. PerMANOVAs were performed with a Euclidean distance matrix and 999 permutations, using the function Adonis in the Vegan package (Oksanen et al. 2011). Non-metric multidimensional scaling was used to visualise the multivariate patterns, using the vegdist function in vegan to create a distance matrix from the GC-MS data then the isoMDS function in the MASS library. There is no post-hoc test available for PerMANOVA using Adonis so individual models were performed to investigate the relationships between progesterone and the scent profile for each otter.

Scent profile richness (the number of chromatogram peaks) was used as a measure of scent profile complexity. To test whether scent complexity and progesterone level were associated for each otter, data were analysed in a general linear model (GLM) using a Poisson family and log link because scent complexity was measured as count data (the number of chromatogram peaks). The number of chromatogram peaks did not include trace compounds (<0.1%). Inspection of the residuals confirmed that the assumption of normality of residuals was not violated. All statistical analysis was carried out in R version 2.13.1 (R Development Core Team 2011).

5.3. Results

5.3.1 Individual identity

After removal of rare compounds (those occurring in <3 spraint samples), a total of 162 compounds were found across all 47 spraint samples. By comparison to the NIST library, 90 were tentatively identified (Appendix 1). Samples varied in the number of compounds detected (Figure 5.1.) (mean±SD: 51±14.28). Samples from Otter Zentrum in Germany (mean ± SD: 66±8.01) had significantly more compounds than all other samples (mean± SD: 44±11.04; $t(45) = -6.73$, $p<0.001$).

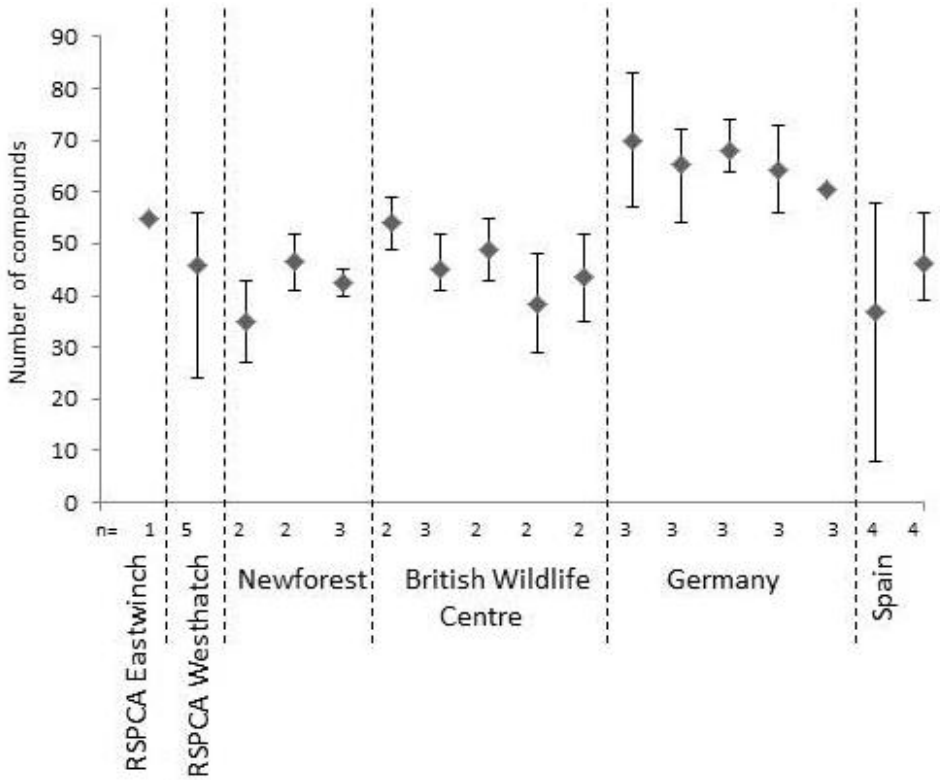


Figure 5.1. Average number of volatile compounds in spraints from 17 otters showing differences between some locations. Error bars indicate range and n is given on x axis.

There were significant differences in scent between individual captive otters ($F_{pseudo}(16, 30)=1.34$, $R^2= 0.42$, $p=0.002$). It was not possible to visualise these differences using nMDS; the stress value was very high with two dimensions (stress=17.27) (Figure 5.2.a) and 23 dimensions were needed to reduce the stress value below one (stress=0.94). Plotting the first two of these 23 dimensions did not improve the visualisation of the PerMANOVA result (Figure 5.2b). These plots did, however, show clusters by country of origin, therefore the samples from the UK were analysed separately, first removing any compounds from that dataset that were not present at all in UK samples. The UK was chosen because it had the biggest sample size. This resulted in 119 compounds across 24 spraint samples from 10 otters. There were significant differences in scent between individual captive otters from the UK ($F_{pseudo}(9,15)=1.22$, $R^2=0.44$, $p=0.041$). These individual differences were slightly clearer than when analysed with the whole dataset, although the nMDS stress value was still high with two dimensions (stress=17.76) (Figure3a) and 13 dimensions were needed to reduce stress below one (stress=0.52) (Figure3b). There was also some clustering by location.

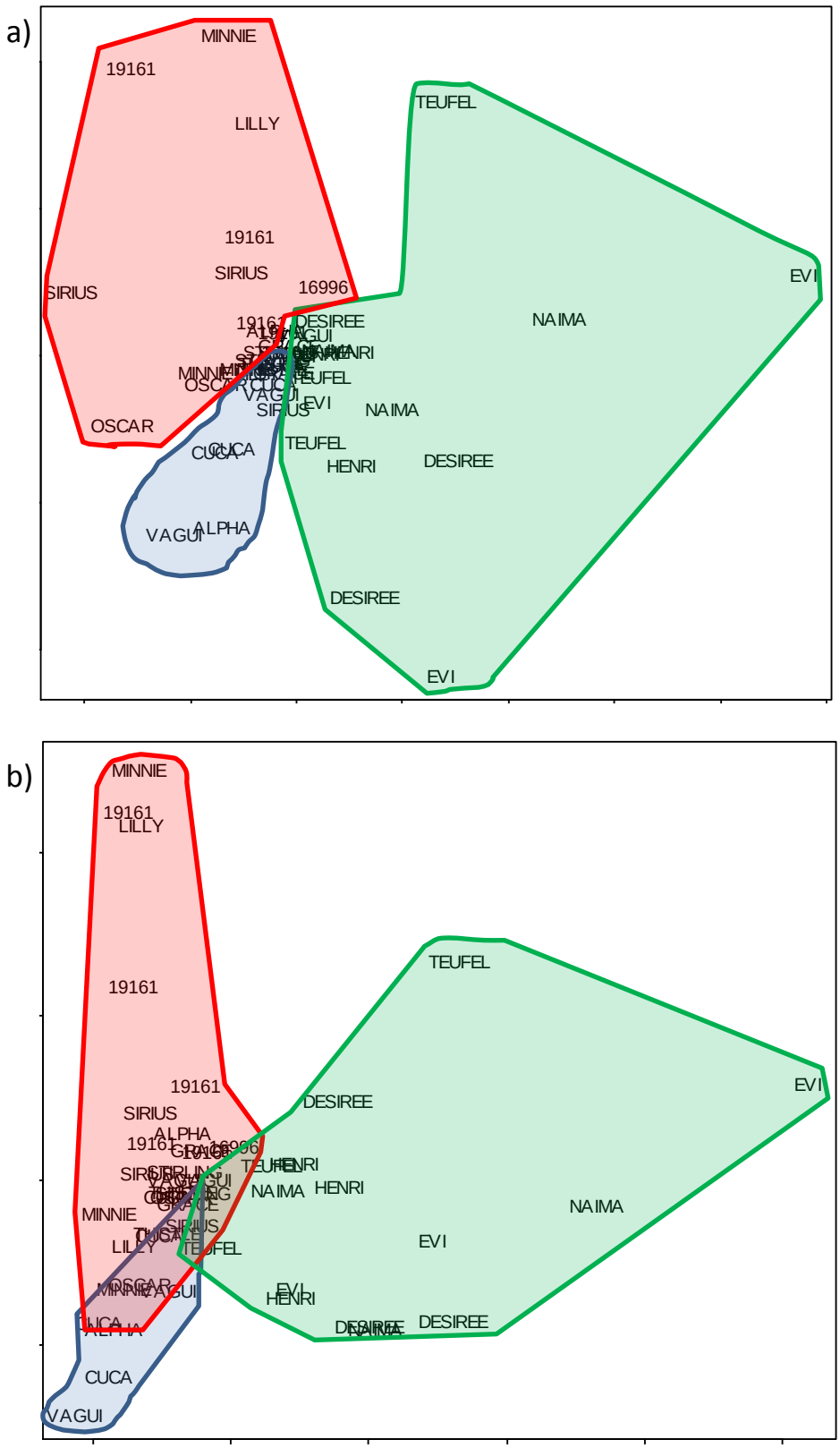
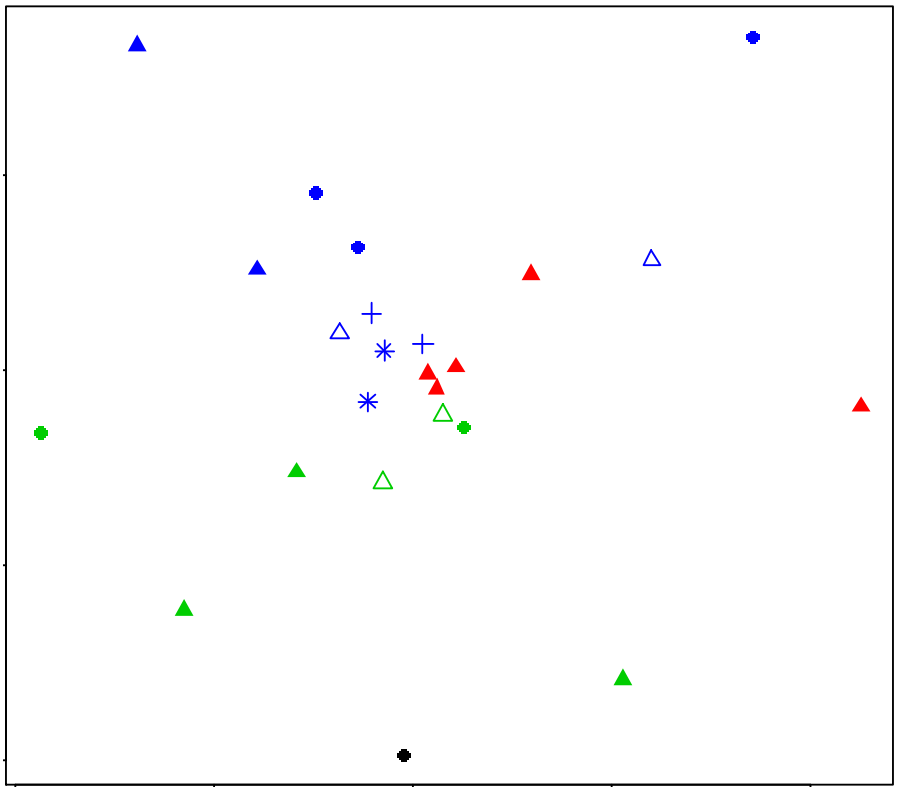


Figure 5.2. Multidimensional Scaling Plots of otter spraint scent showing groupings by country of origin (a) 2 dimensions, stress=17.27, (b) 23 dimensions, stress=0.94. Repeat samples from 17 otters labelled with otter name or identity number. Coloured outlines show clustering by otter country of origin; —UK —Germany —Spain.

a)



b)

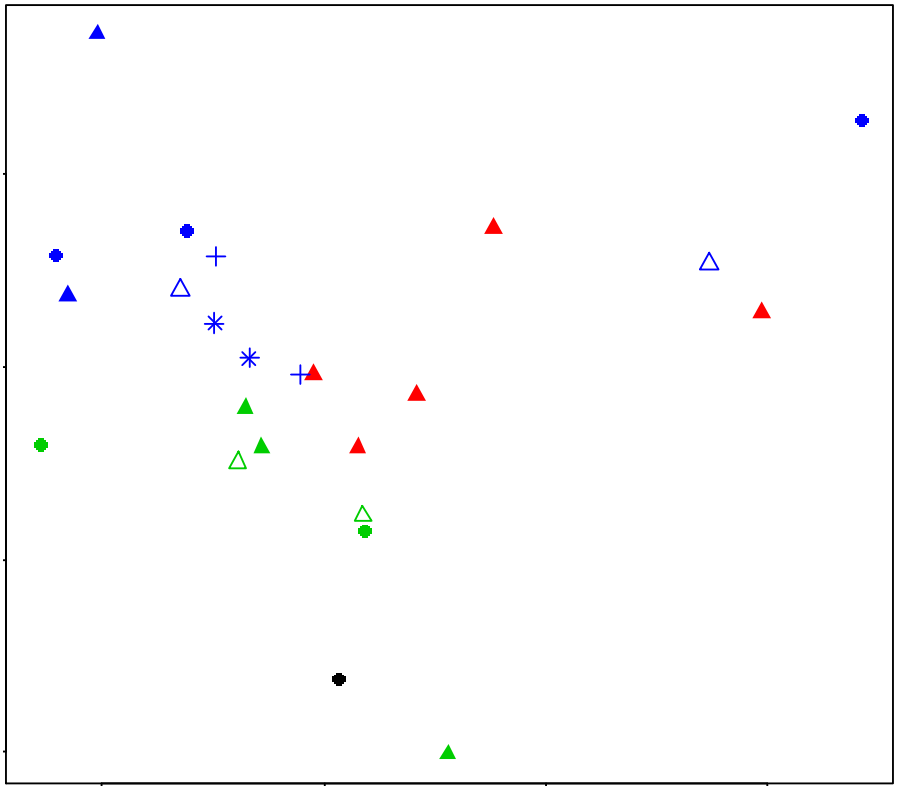


Figure 5.3. Multidimensional Scaling Plots of otter spraint scent indicating similarity between samples taken from the same otter. (a) 2 dimensions, stress=17.76, (b) 13 dimensions, stress=0.52. Symbols indicate repeat samples from 10 otters, colours indicate four different centres where otters were housed.

Three or more samples were obtained from three otters from the UK. In the nMDS plots it appears as though some samples are more similar than others. This was not explained by the date when samples were collected. Three samples were collected from one otter, Minnie, in August, October and November 2009 shown in Figure 5.3 as filled blue circles. The two samples that appeared close together in the nMDS plots were the August and November samples, and the chromatograms from these samples are more similar than the October sample (Figure 5.4.).

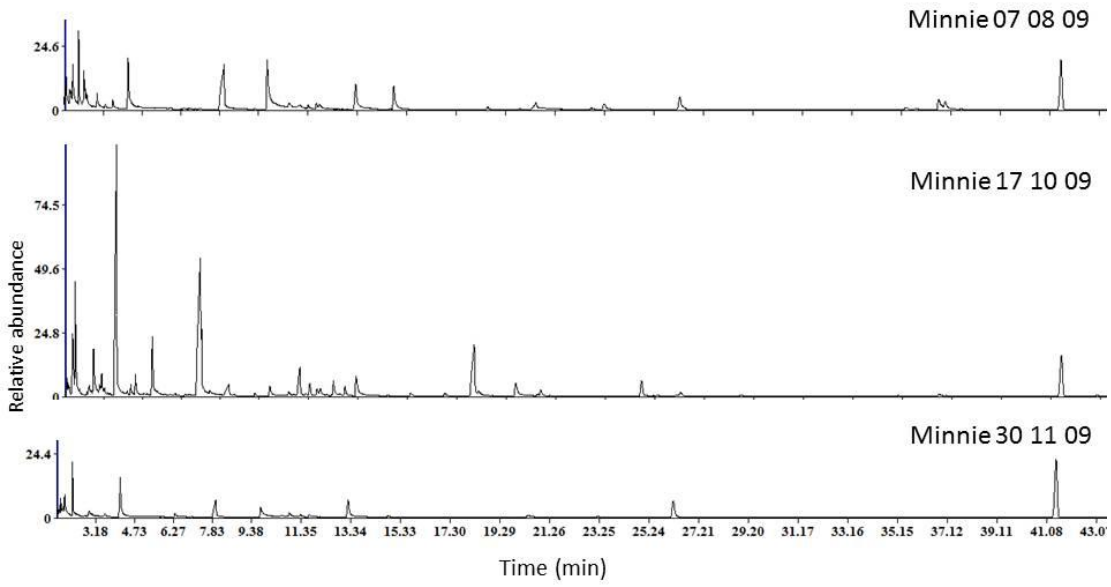


Figure 5.4. Total Ion Chromatograms from GC-MS analysis of three spraint samples collected from an adult female otter (Minnie).

5.3.2 Female reproductive hormones

As no oestrogen metabolites were found in the spraint, reproductive hormone analysis focused on progesterone metabolite. Progesterone in spraints varied considerably, ranging from 7.20 to 756.64 ng g^{-1} dried spraint, with a mean basal level of 63.91 ng g^{-1} and mean peak level of 323.39 ng g^{-1}. The progesterone levels over one month fluctuated in three otters (mean±SD: 251.66±131.16) and did not in the remaining three otters (mean±SD: 59.60±33.65) (Figure 5.5.). The three otters that did have fluctuating progesterone levels (Lotta, Baruna and Volga) were all housed at the same

centre in France. The spraints from one female otter (Lotta) showed considerable variation and so further progesterone analysis was conducted over an additional two months (Figure 5.6.) but during this time there was no regular pattern that would indicate stages of the oestrous cycle.

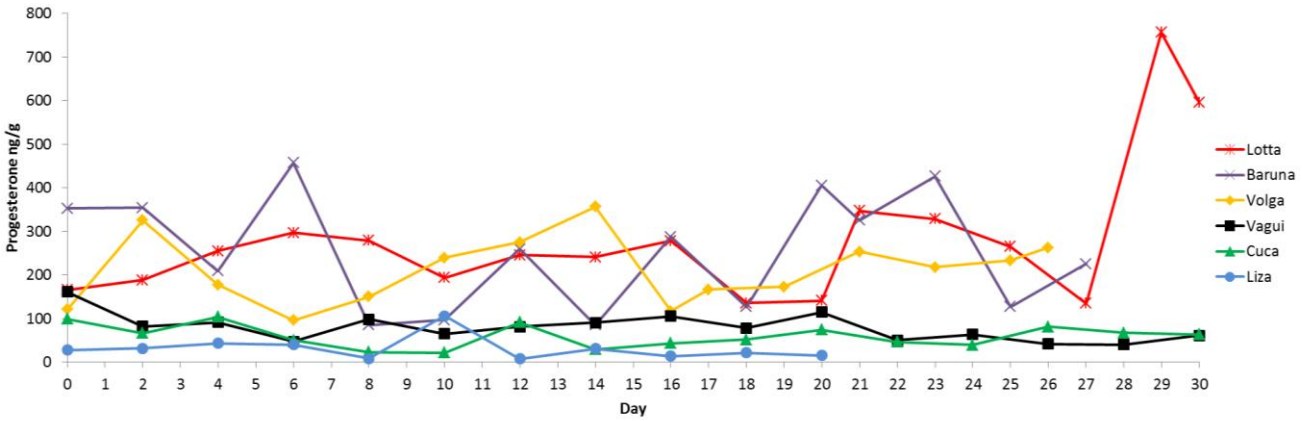


Figure 5.5. Progesterone profile from the spraints of six female Eurasian otters for which scent data were also collected. Day 0 was the first day that spraint was collected and subject to progesterone analysis. Note actual dates varied.

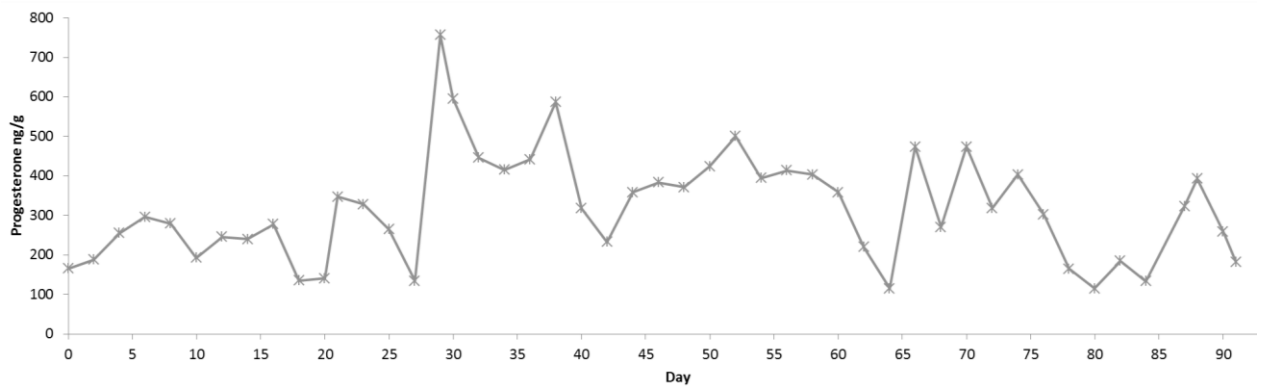


Figure 5.6. Progesterone profile of one female captive otter (Lotta) over 3 months.

A total of 284 VOCs were found across all 76 spraint samples, 138 of which were provisionally identified by comparison to the NIST library. There was a significant positive correlation between progesterone and the number of VOCs ($r_s(72)=0.52$, $p<0.001$) (Figure 5.7). A GLM showed that this association was significant for only three otters; Cuca ($z=3.17$, $p=0.0015$) Liza ($z=2.64$, $p=0.008$) and Lotta ($z=7.52$, $p<0.001$) (Figure 5.8). The association between progesterone and number of VOCs

remained for Lotta even when the outlier (88 VOCs and 442ng g^{-1} progesterone) was removed ($z=4.14$, $p<0.001$). Some variation in the scent profile was significantly explained by otter identity ($F_{pseudo}(5, 68)=2.11$, $R^2=0.13$, $p=0.001$) and progesterone level ($F_{pseudo}(1, 72)=2.76$, $R^2=0.03$, $p=0.001$), but not otter location. The interaction between otter identity and progesterone level also significantly explained variation in scent profile ($F_{pseudo}(5, 68)=1.80$, $R^2=0.11$, $p=0.03$) indicating the relationship between VOCs and progesterone was different between otters. Progesterone significantly explained some variation in scent profile for Cuca ($F_{pseudo}(1, 13)=1.56$, $R^2=0.12$, $p=0.046$) at the 0.05 significance level and for Lotta ($F_{pseudo}(1, 13)=1.51$, $R^2=0.12$, $p=0.098$) at the 0.1 significance level. There was no association between progesterone and scent profile for any of the other otters (Liza ($F_{pseudo}(1, 8)=2.07$, $R^2=0.21$, $p=0.14$), Baruna ($F_{pseudo}(1, 10)=0.62$, $R^2=0.06$, $p=0.99$), Volga ($F_{pseudo}(1, 13)=1.14$, $R^2=0.12$, $p=0.24$), Vagui ($F_{pseudo}(1, 12)=1.18$, $R^2=0.09$, $p=0.29$)).

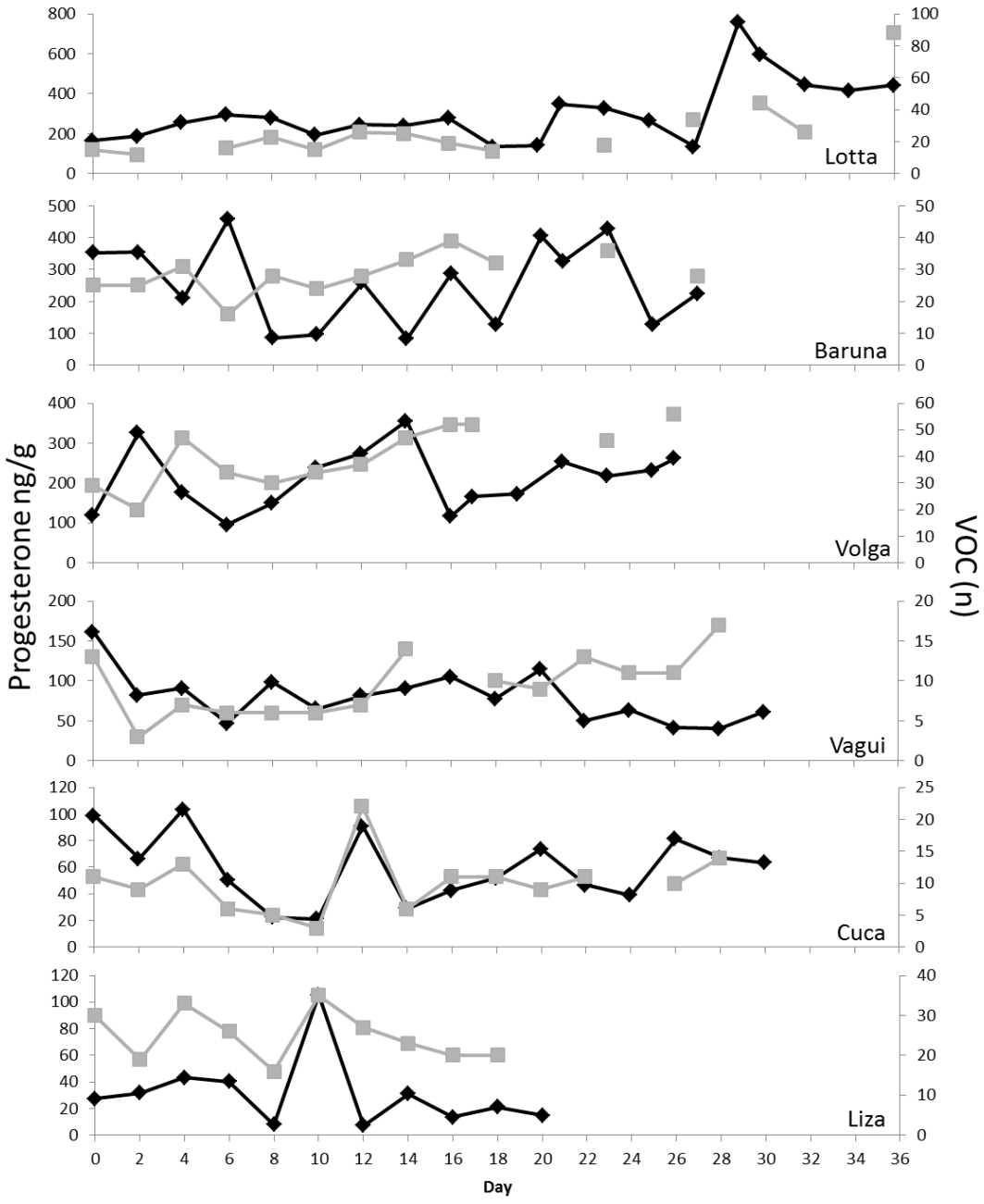


Figure 5.7. Profiles of progesterone (black) and number of VOCs (grey) found in spraints collected from six otters.

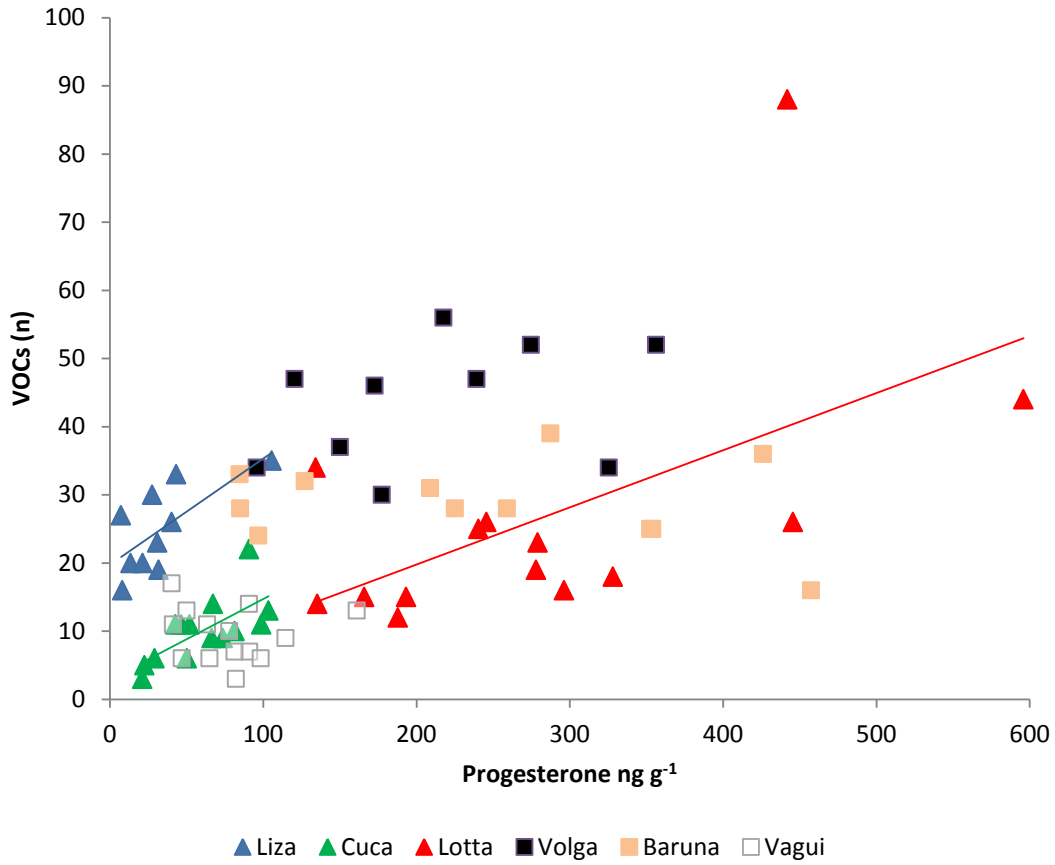


Figure 5.8. Correlation between number of volatile organic compounds and progesterone level in spraints from six otters. Lines of best fit are given for otters for which this relationship was statistically significant.

5.4. Discussion

The results from the analysis of spraints collected from 17 captive otter analysed at Cardiff University, and from six otters analysed at Bristol University show some variation in VOCs from otter spraints are significantly explained by the individual otter that deposited the spraint. This suggests that there is an individual scent signature in Eurasian otter spraint, as there is with other mustelid species (Brinck et al., 1978; Buesching et al., 2002b, c; Erlinge et al., 1982; Zhang et al., 2003, 2005). Only 42% of variation was, however, explained by otter identity, indicating that as well as between individual variation, there is also a lot of within individual variation. Scent profiles also vary significantly between spotted hyaena individuals but they also change in composition over time (Burgener et al., 2009). Within and between variation in the scent profiles of mammals is not surprising given the complexity of

scent profiles and the variety of variables that could influence scent or be communicated by scent.

In the first experiment spraints from otters housed in the same centre were similar to each other. This phenomenon was also observed by Davies (2009), who suggested common diet as a potential explanation. The diet of the otters in the present study is unknown but given the results in Chapter 3 it is unlikely this grouping by centre is due to diet. An alternative possibility is that differences in collection methodology between centres influenced the data. Spraints were collected by keepers, and samples collected in Germany were stored in plastic zip lock bags rather than more robust plastic tubes, as requested. These samples had significantly more VOCs than samples from other locations and an increase in complexity is associated with decaying samples (see Chapter 3). In the second experiment, otter location did not explain variation in VOC profiles. Group differences in scent have been found in group living species such as badgers (Davies et al., 1988; Buesching et al., 2002b) who rub anogenital regions together to share scent (Gorman et al., 1984; Buesching et al., 2003). There is some debate as to the extent of solitary living of Eurasian otters but it would not be possible for the otters in this study to share group scent by a similar mechanism because they were all housed alone. Although not necessarily closely related, it is reasonable to assume that, in this study otters from the same centre are more likely to be related than animals from different centres, given the different countries of origin and isolation by distance in population genetics. Genetic similarity in scent has been found in lemurs and mice (for review see Hurst and Beynon, 2010) and differences in scent have been reported between subspecies of beavers (Rosell and Steifetten, 2004). For the samples analysed in the first experiment (at Cardiff University) other possible sources of variation include sex or age as found in Chapter 4. Other possible sources of variation include substrate, time between deposition and collection, time between collection and analysis, health status and reproductive status.

Samples collected closer together in time were not more similar to each other than samples with a longer sampling interval, exemplified by one individual in particular. Similarly, an individual badger's scent was more similar in the same season across

years than in the same year across seasons (Buesching et al., 2002c). This suggests the variation within an individual may be due, at least in part, to variation in reproductive hormones, hence the parallel investigation of VOCs and reproductive hormones in the second experiment. The second experiment controls for any potential variation in age or sex as all were from adult female otters. Despite this, in the second experiment even less variation was explained by individual identity; just 13%. This difference between experiments may be explained in part by the difference in number of repeats; many more samples per individual were analysed in the second experiment.

The progesterone levels recorded here in three non-pregnant female otters frequently exceeded 220ng g^{-1}, calling into question the previous assumptions about progesterone levels indicative of pregnancy (Tschirch et al., 1996; Kalz et al., 2006), and the conclusions about wild otter populations based on these assumptions. Basal progesterone levels in female North American river otters (572.46 ±12.98 ng g^{-1}) and Asian short clawed otters (254.07 ±39.92 ng g^{-1}) are higher than those recorded here and estradiol can be measured in the faeces from these species throughout the year (Bateman et al., 2009). Bateman et al. (2009), however, were not able to determine oestrous from faecal oestrogen levels in either of these species.

Three of the otters had progesterone levels that were consistently at lower levels than the other three otters and fluctuated less. It is likely that these otters were not cycling as one was lactating, one had been paired with a male several times but failed to breed and one was possibly not yet sexually mature at only 2.2 years old (females are estimated to reach sexual maturity in their second year, Hauer et al., 2002) and was the only otter that was housed at a centre that did not also keep male otters. Oestrous cycle length varies between mammal species and is characterised by a peak in oestrogen, followed by a rise in progesterone. Even in the three otters that had higher progesterone levels, the patterns recorded do not exhibit regular cycles and the absence of oestrogen metabolites supports the possibility that they were not cycling.

The fact that no oestrogen metabolites were detectable in the present study may suggest that these Eurasian otter females were not reproductively active and that the

presence of a male is needed to stimulate reproductive activity (Bill Holt, pers. comm.). This is consistent with previous suggestions that Eurasian otters are induced ovulators (Broekhuizen, 2007; Jorga et al., 2004). Mustelids vary in reproductive traits with flexibility in oestrous induction within species and some reproductive peculiarities; although most are mono-oestrous or seasonally poly-oestrous, and oestrous can last for months (Amstislavsky and Ternovskaya, 2000). Although otters have been found to be pregnant at any time of year (Chadwick and Sherrard-Smith, 2010: Hauer, 2002: Heggberget and Christensen, 1994), there is still the possibility that they could be mono-oestrous or seasonally poly-oestrous. Prolonged sperm survival in the female reproductive tract and induced ovulation (commonly found in mustelids; Amstislavsky and Ternovskaya, 2000) and delayed implantation (observed in otters; Brokhuizen et al., 2007) could allow for this possibility. Monitoring of faecal hormones over at least one year would be necessary to detect this. All but one otter (Liza) had males housed adjacent to them, but perhaps direct contact is necessary or these males were too familiar to induce reproductive activity. Breeding success of Eurasian otters is enhanced if male and females are kept separately (Melissen, 2000) and female otters that have been housed with the same male for several years and failed to breed have subsequently had cubs when introduced to a different male (Jason Palmer, Newforest Wildlife Park, pers. com.). The male effect of inducing ovulation is well documented in livestock (Martin et al., 1986) and this affect is increased if the male is unfamiliar (Hawken and Beard, 2009).

There was a significant positive correlation between progesterone concentration and the complexity of scent profiles for three of the otters. In addition, progesterone concentration significantly explained a small amount of variation (3%) in VOC abundances. This is consistent with associations between stage of reproductive cycle and VOCs in other species (Ma and Klemm, 1997; Kumar et al., 2000; Rajanarayanan and Archunanand, 2011; Andreolini et al., 1987).

The three otters that had a significant correlation between progesterone concentration and VOC complexity were not the same three otters that had overall higher progesterone concentrations. There is no clear difference between otters whose scent

complexity increased with progesterone and those where there was no relationship between these two variables, in terms of location, adjacent males, or reproductive history. In this second experiment location did not explain any of the variation in scent profiles. Those which exhibited a correlation were all younger (2.2, 3 and 3 years old) than the other three otters (3.4, 4 and 4 years old) but this is not a big difference in age. Time of year does not explain this difference either as all samples were collected at the same time of year (between April and May) apart from one individual (Liza) whose samples were collected one month later. It can not be ruled out that the difference is in stage of the reproductive cycle; our failure to detect oestrogen metabolites could be because they are not present in faeces, rather than they were not present in the otters. It could be hypothesised that samples varied in quality (time between deposition and storage at -20^{o}C), potentially affecting our ability to detect both steroid metabolites and VOCs, thus explaining a relationship between progesterone concentration and number of VOCs. This, however, is unlikely because of the positive correlation found in this chapter; degradation results in less faecal steroid metabolite (Moestl et al., 1999; Wasser et al., 1988) and more VOCs being measured (Chapter 3), and would therefore lead to a negative correlation. Additionally, the samples used for faecal hormone analysis and those used for VOC analysis were separated by one day and so are subject to different fluctuations in time between deposition and storage at -20^{o}C.

Only one otter at each of the three locations had a positive correlation between progesterone and VOC complexity. If VOC and progesterone correlation indicates reproductive activity, then the fact that only one female at each location shows this pattern, might suggest there is reproductive suppression between females, although clearly the sample size is insufficient to draw any conclusions. Stress experienced by subdominant females when in contact with more dominant females can disrupt the reproductive cycle. While most examples of reproductive suppression come from group living species (for review see Stockley and Bro-Jørgensen, 2011), there is evidence from the solitary golden hamster (Huck et al., 1983, 1988a, b).

Some of the remaining unexplained variation in scent profiles in the first experiment may be due to hormonal variation as observed in the second experiment. There was, however, unexplained variation between scent profiles in both experiments. This extra variation in scent profiles may be due to variables that were not investigated here, for example; substrate, time between deposition and collection, time between collection and analysis, and health status (age and sex were controlled for in the second experiment). Individuality can be communicated in urine volatiles (Singer et al., 1997) so it is possible that volatiles from urine are obscuring individual signatures in some spraint samples or conversely that ownership signals are clearer from samples that contain urine. Otters have been observed to urinate on top of spraint (Kruuk, 2006) but it is unknown whether the otters urinated on any of the samples analysed here. There could be other sources that give a clearer picture of individual identity. Otters frequently make direct contact with spraints (Chapter 2). This suggests that they might communicate via non-volatile compounds, as in mice (Hurst et al., 2001: Nevison et al., 2003).

The results presented here do suggest that individuality is present in otter scent VOCs, but there is insufficient resolution to allow prediction of identity from unknown samples (as would be necessary to monitor wild populations). Clearly many variables influence otter scent profiles. This is also true for other species which is why Hurst and Beynon (2010) argue for selective assessment of specific compounds within scent. Selection of compounds only found in a reference sample has successfully been used to identify human individuals from scent samples (Curran et al., 2010). To apply this method to otters would require trapping individuals to gain a reference sample, which is not appropriate for monitoring populations. Uninformed selection of compounds that correlate with the target variable (identity, genetic relatedness) could result in Type 1 error. Any selection (or deselection) of compounds should instead be based on systematic evaluation of other sources of variation in VOCs including sex, age, reproductive status, degree of decay etc.

The results presented here suggest individual scent fingerprints exist in the spraints of Eurasian otters and that scent profiles of female otters are influenced by progesterone.

Further combined monitoring of steroid metabolites and VOCs from spraints for a minimum of one year would be needed to fully evaluate the reproductive cycle of *Lutra lutra* and to see if this relationship between VOCs and progesterone is maintained through oestrous. In addition experimentally introducing unfamiliar males could test if the male effect of inducing ovulation exists in otters.

Chapter 6
Spatial analysis of scent

6.1. Introduction

In wild populations, individuals that are closer together geographically are more likely to be related than those further apart. Geographical separation can lead to genetic differences, but the impact that this has on scent has received very little attention. On a large scale there are differences in scent between species (e.g. canids, Burnham et al., 2008) and some evidence from beavers that suggests there are scent differences between subspecies (Rosell and Steifetten, 2004). On a much smaller scale, there is growing evidence that relatedness may be communicated in scent, for example, within a captive population of lemurs (Charpentier et al., 2008), within beaver families (Sun and Müller-Schwarze, 1998b) or between bat colonies within 15km of each other (Safi and Kerth, 2003). Differences in urine VOCs between mice that differ only in genes of the major histocompatibility complex (MHC) have been reported (Willse et al., 2005). The communication of genetic relatedness through scent on this smaller scale has been suggested to function in kin recognition and inbreeding avoidance or nepotism (Charpentier et al., 2008). There is further recent evidence of a correlation between genetic similarity and similarity of volatile chemicals in lemur gland secretions (Boulet et al., 2009) but more work is needed to confirm a role in recognition (Hurst and Beynon, 2010).

There has been very little research within the field of chemical ecology to examine if genetic differences on a large scale within the same species have any impact on scent composition. An exception is rabbit populations, which have been shown to differ in their scent when separated by more than 100km (Hayes et al., 2002). There is genetic sub-structuring in the UK otter population relating to geographical location, probably as a result of re-colonisation from small founder populations following severe declines in otter numbers across the UK during the 1960s (Hobbs et al., 2011). In this chapter I test the hypothesis that Eurasian otter scent signals vary spatially, at either the regional or the local scale. Firstly, the study considers whether variation in otter scent is associated with genetic sub-structuring of populations in the UK, based on the four regions defined

by Hobbs et al. (2011). Secondly, the study considers whether scent signatures of field collected spraints vary between sites within a catchment, and assess whether a combination of geographic location and scent difference can be used to infer individual identity.

6.2. Methods

6.2.1 Spatial analysis of scent gland secretions from carcasses

Scent gland samples taken from otter carcasses and analysed in Chapter 4 (n=158) were assigned to one of four genetic regions of England and Wales according to the population substructuring established by Hobbs (2011). Some samples originated outside of these four genetic regions, resulting in a reduced sample size of 123 (Figure 6.1). These four regions were North England, Central England, Wales and the borders and the South West. PerMANOVA (using a Euclidean distance matrix with 999 permutations) was used to investigate if these genetic regions explained any variation in the scent data (432 VOCs) while controlling for age, sex and reproductive status. PerMANOVA was performed using the Adonis function in R; post-hoc tests do not exist for the Adonis function, so separate tests were performed on male and female data. To test whether this was based purely on distances between samples, a Mantel test was used. This tested for correlation between two dissimilarity matrices: geographical location where the otter was found (x and y coordinates) and all scent data (432 VOCs). The Mantel test was based on Pearson product moment correlation with 999 permutations.

6.2.2 Spatial analysis of spraint scent from a wild population

Field surveys for otter spraint were conducted on the lower river Wye and adjacent Gwent levels in Monmouthshire, Wales. Every bridge over the water bodies was visited in a pilot study to assess the suitability of spraint sites; sites that were inaccessible or too dangerous to inspect and those with no suitable sprainting areas were excluded. This followed a recommended protocol found to be practical on several rivers (Chanin, 2005). To reduce large distances between sites, additional sites were searched in the pilot study and added if otter signs were found, resulting in 29 sites. Underneath each bridge, and up to approximately 50 m either side, was searched. The survey was

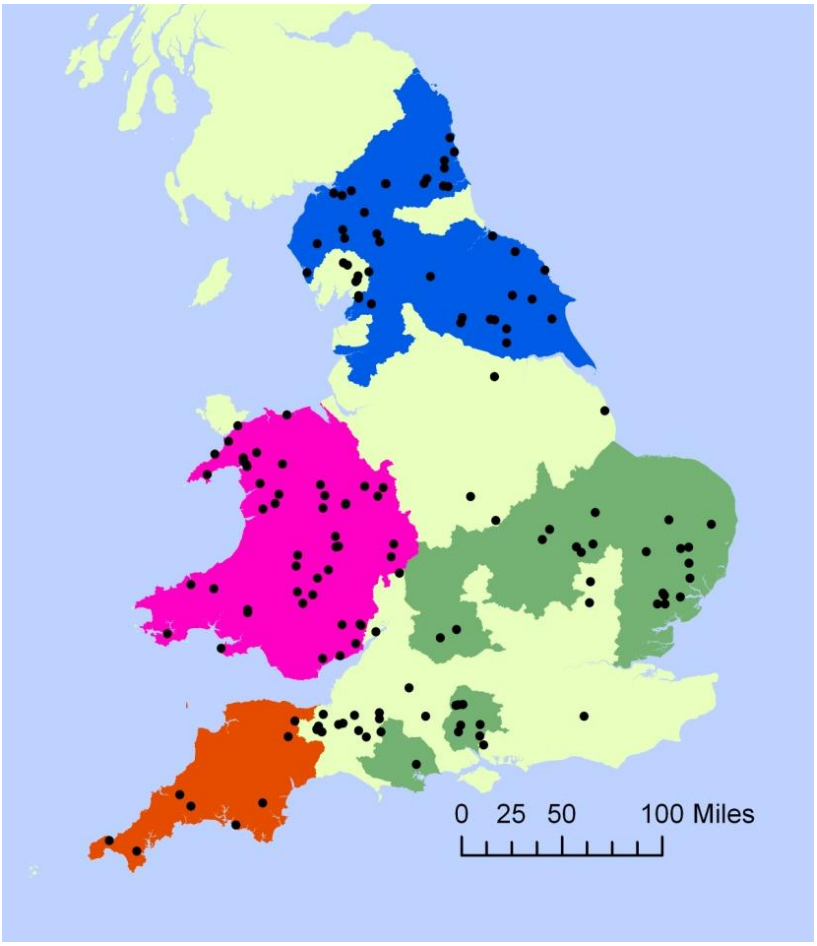


Figure 6.1. Distribution of otter anal gland samples across England and Wales used to investigate spatial variation in scent profiles. Four regions with genetically distinct otter populations (identified by Hobbs et al., 2011) are indicated in pink, orange, dark green and blue.

conducted on 5th May 2010. Any spraint or anal jelly found were assessed for freshness and fresh spraint (wet, not dry and crumbly) was collected into 7ml sterile plastic tubes, using disposable gloves and if necessary disposable wooden sticks (each only used once). A section rather than the whole sample was collected due to ethical concerns about removing scent signals (Yoxon, 1998). In a preliminary experiment hierarchical cluster analysis of spraint subsections revealed that similar GCMS results were obtained from subsections (data not presented here). Samples were stored in a cool bag with ice packs for up to 8 hours and then stored for five months at -20°C until analysis.

Samples were analysed according to the methods developed in Chapter 3 and VOCs were matched to the scent library created in Chapter 5. A hierarchical cluster analysis (based on Euclidean distance) was performed on the VOC data to examine if spraints

grouped by location within the survey area. A Mantel test (based on Pearson product moment correlation with 999 permutations) was used to test for correlation between geographical location where the spraint was found and VOC data. All data analysis was conducted in R (version 2.13.1).

6.3. Results

6.3.1 Spatial analysis of scent secretions from carcasses

Significant differences in scent gland VOCs were found between sexes ($F_{pseudo}(1, 121)=3.57$, $R^2=0.03$, $p<0.001$), age classes ($F_{pseudo}(2, 120)=3.36$, $R^2=0.05$, $p<0.001$) and reproductive status' ($F_{pseudo}(4,118)=1.94$, $R^2=0.06$, $p=0.002$) as found in Chapter 4. There was no significant difference in scent between genetic regions ($F_{pseudo}(3, 119)=1.14$, $R^2=0.03$, $p=0.17$) but the interaction between genetic region and sex was significant ($F_{pseudo}(3,119)=1.62$, $R^2=0.04$, $p=0.038$) indicating that genetic differences are expressed differently by males and females. The interaction between genetic region and age was not significant. There were significant differences between the genetic regions for females ($F_{pseudo}(3,73)=1.42$, $R^2=0.05$, $p=0.04$) and near significance for males ($F_{pseudo}(3, 42)=1.32$, $R^2=0.08$, $p=0.09$). This difference between genetic regions was not based on just geographical distance between samples because there was no correlation between spatial distance matrix and scent distance matrix using all samples ($r=0.02$, $n=158$, $p=0.13$), adult samples ($r=0.02$, $n=86$, $p=0.33$), adult male samples ($r=0.07$, $n=27$, $p=0.11$) or adult quiescent female samples ($r=-0.04$, $n=24$, $p=0.67$).

6.3.2 Spatial analysis of scent from a wild population

A hierarchical cluster analysis was performed on the scent data from the spraints and anal jellies collected in Monmouthshire (Figure 6.2) and although some spraints which were from similar geographical locations clustered in scent (e.g. 19 and 23), many did not (samples are identified in the cluster analysis with a number, the geographical location of these are shown in Figure 6.3). Furthermore some samples grouped in the cluster analysis which were unlikely to be from the same otter due to lack of connected water courses (e.g. 4 and 13). There was no correlation between the spatial distance matrix and the scent distance matrix ($r=0.10$, $n=25$, $p=0.13$).

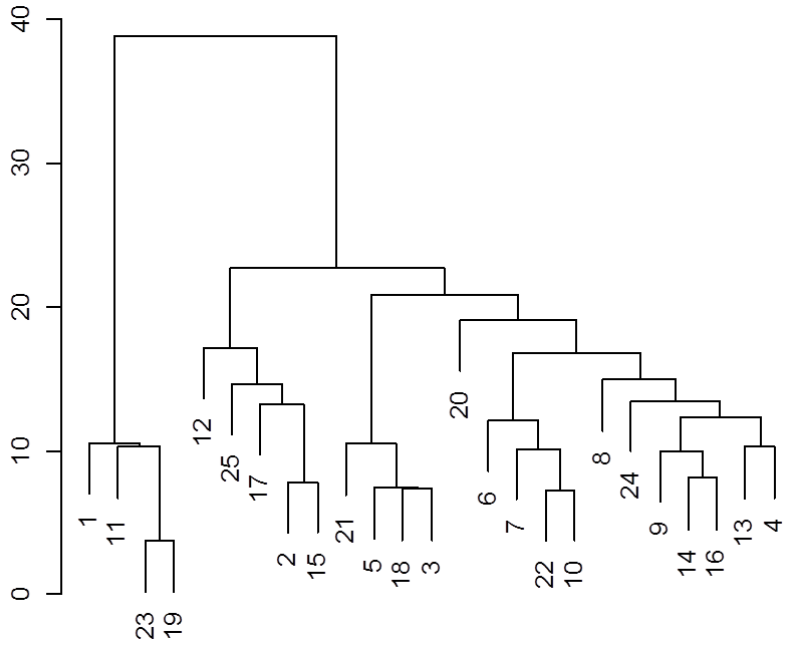


Figure 6.2. Hierarchical cluster analysis of volatile compounds from otter spraints and anal jelly collected on the same day in Monmouthshire. Samples were analysed using solid phase microextraction and gas chromatography mass spectrometry. Numbers indicate sample number and the geographical locations of samples are shown in Figure 6.3.

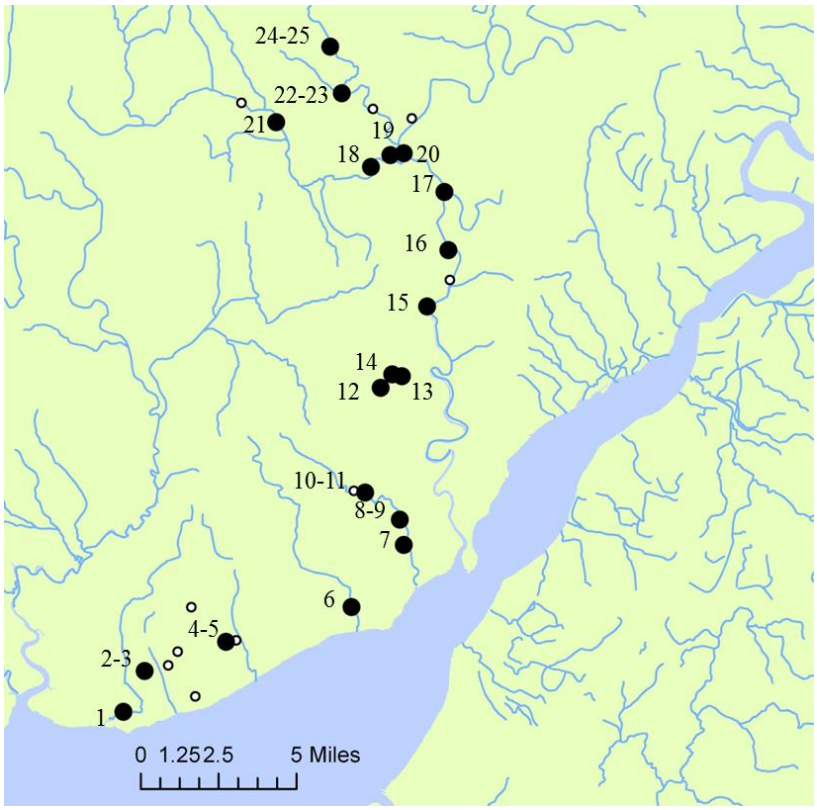


Figure 6.3. Location of spraint or anal jelly found on the River Wye and Gwent levels in May 2010. Filled circles are sites where samples were found, open circles are sites where no otter signs were found.

6.4. Discussion

Significant differences in the anal gland scent of otters were found between the four genetic subpopulations in the UK, indicating that scent signals are differentiated at a regional scale. These four genetic subpopulations are the result of the UK otter population decline in the last century, and recovery from small and spatially separated surviving populations (Hobbs et al., 2011). This finding is in agreement with the growing body of evidence of scent similarity and genetic similarity mostly derived from studies of relatedness at an individual level (Sun and Müller-Schwarze, 1998b; Charpentier et al., 2008; Boulet et al., 2009). Although an association between genetic similarity and scent similarity has been found between bat colonies, the authors did not find the same association within colonies (Safi and Kerth, 2003). These scent differences are more likely to reflect colony membership rather than genetic similarity. The present results appear to be the first evidence of genetic and scent similarity in any species at the population genetics level. Additionally most studies in this field of research are on captive populations; this Chapter is one of very few studies on free-ranging wild populations.

Differences in rabbit odours have been found based on geographical distance (Hayes et al., 2002). For otters, however, the present indicate that geographical distance alone is not a good measure of difference in scent as there was no correlation between geographical distance and scent distance. An otter's scent is associated with its location but only in terms of the genetic subpopulation it belongs to. Some geographical distances between otter locations may not be relevant to relatedness, for example there may be a very short distance between otters but a migration barrier such as a large road prevents breeding between them.

Other research has not found an association between relatedness and VOCs, for example in spotted hyenas (Burgener et al., 2009) and North American river otters are reported to not discriminate between scents depending on the relatedness of the donor conspecific (Rostain et al., 2004). Both these studies only tested a single sex. The present results indicate that the genetic differences are expressed in scent differently between males and females. There was a significant interaction between genetic

subpopulations and sex; differences between the genetic subpopulations were significant for females and near significant for male otters. Similar to other variables tested in other Chapters, only a small amount of variation in the scent was explained by the genetic sub populations. It is possible that variation caused by other variables prevented Burgener et al. (2009) from finding differences in scent associated with relatedness. Alternatively, the results presented here for female otters may be clearer than for male otters due to the larger number of females (n=77) than males (n=46) in the dataset. Burgener et al. had a smaller sample size for their tests (although the total sample size was 50, this consisted of three clans which were analysed separately).

On a small scale (within Monmouthshire) there was no correlation between spraint location and scent. Although differences in spraint scent between individual otters were found in Chapter 5, the resolution of these differences was not strong enough to make predictions from unknown spraints collected in the wild. It is not surprising that clear differences based on location and scent were not found. Those samples that are similar in scent and location are likely to be from the same otter but it is not possible to confirm this. Similarities in scent between samples which are thought to be geographically separated due to lack of connected water courses are likely to be from different otters but of the same age or sex group. Samples which are close in location but very different in scent may indicate overlapping territories as has been found through genetic analysis of otter spraint (e.g. Kalz et al., 2006) or the marking of territory edges. Future research should incorporate genetic analysis of spraint, in order to independently verify scent differences. Additionally, genetic analysis of tissues and scent analysis from glands originating from the same animals could be used to further test the hypothesis that scent differences are associated with relatedness.

In summary, it is not possible to use scent profiles and geographical location of spraint samples to infer individual otter identity. The main finding of this study is that genetic subpopulations of *Lutra lutra* differ in the odour profiles of their anal gland secretions.

Chapter 7
General discussion

Scent communication is the most important mode of communication to many mammalian species (Albone 1984; Brown and Macdonald, 1985; Wyatt, 2003) but is often overlooked because of the dominance of visual and vocal communication in our own species. Knowledge of scent communication can assist conservation (Campbell-Palmer and Rosell, 2011), or can be used to manage problem individuals or populations (Müller-Schwarze, 1990; Shumake, 1977). The Eurasian otter, *Lutra lutra*, is one of the largest carnivores extant in Great Britain but suffered huge population declines in the last century due mainly to persistent organic pollutant usage (reviewed by Chanin, 2003). Methods used to monitor populations lack the specificity that would allow population size estimation and research on population dynamics. Despite wide discussion on the limitations of spraint collections (Jefferies, 1966; Mason and Macdonald, 1987; Kruuk and Conroy, 1987; Wilson and Delahay, 2001), speculation about the function of spraint (Kruuk, 1992, 2006; Gosling, 1982) and general interest in otters, little progress had been made in understanding what information these signals convey. Although significant progress has been made in communication research over the last few decades, both in terms of methods used and taxa studied, very little information was available for any of the thirteen otter species worldwide (Chapter 1). In this study I employed a combination of chemical analysis, experimental behavioural tests and field collections of scent marks to examine scent communication in the Eurasian otter, *Lutra lutra*. This study aimed to describe chemical differences in otter scent relating to otter identity and test whether this could be used to identify otters from spraint collected in the wild. In this chapter I review the main findings firstly in terms of spraint analysis as a research tool and, secondly, their relevance to otters. Finally, I discuss the limitations of this research and recommendations for future research.

7.1 Scent analysis methods

Solid phase micro extraction (SPME) is the method of choice for analysis of complex mixtures of volatile or semi-volatile compounds (Heath and Dueben, 1998) and this study presents the first research on a mustelid species using this technique. There is,

however, considerable variation within this technique. Systematic optimisation of this technique for the analysis of otter scent secretions was detailed in Chapter 3, giving consideration to fiber type, exposure time and sample storage. This vital step in scent communication research tends to be largely unreported in the published literature despite being communicated frequently in other fields of chemical analysis (e.g. Zhu et al., 2009). These parameters may have been tested but it is not possible to ascertain if it is not reported. If these parameters are not thoroughly tested, a suboptimal method may be employed resulting in false negatives. Commonalities may exist in optimal methods for the analysis of animal scent secretions, but this is not possible to assess without reporting method development. Chapter 3 provides a fully optimised method for the analysis of otter scent secretions and recommendations for the parameters that should be tested and reported in optimising methods for chemical analysis of scent secretions from other species.

Method optimisation is not limited to the laboratory methods; the type of statistical analysis employed affects the outcome of research. Statistical methods in chemical analysis of mammalian scent have expanded over the last 40 years. Early research on chemical analysis frequently reported annotated chromatograms with no statistical analysis (e.g. Müller-Schwarze, 1971). This was followed by counts of occurrence (e.g. Rosell and Sundsdal, 2001) or multiple univariate analyses of single compounds (e.g. Zhang et al., 2002a) which, if conducted without a corrected alpha value, is associated with type 1 error (false positives). Some studies have focused on the most abundant components but the compounds that produce the greatest discrimination between groups are not always the most abundant (Willse et al., 2005). If only single compounds are examined, this ignores the potential for differences in scent that are a result of complex mixtures and the ratio of compounds. Sun and Müller-Schwarze (1998) proposed two possible forms of scent coding; differences in the relative abundance (analog coding) and presence/absence (digital coding) of individual compounds. Multivariate analyses combine analog and digital coding, and have been used in this thesis to reveal subtle signaling differences between organisms involving complex mixtures of compounds. Discriminant Function Analysis and PerMANOVA consider most or all of the scent variables, incorporating all of the complexity of the data. These tests may be a closer approximation to how an animal receives the scent than other, simpler analyses.

Although some authors argue for a stepwise approach in analysing scent (Hurst and Beynon, 2010), this method is viewed with caution because of the high occurrence of Type 1 error (Whittingham et al., 2006). In a workshop on methods at the Chemical Signals in Vertebrates meeting in 2011, Berlin it was clear there is no consensus on the most appropriate methods to test for differences or similarities in scent.

The current results (particularly those presented in Chapter 4), suggest that those studies that have not found chemical differences in scent using simple univariate analyses (see Table 1.1), may benefit from re-examination of the data. Similarities can be drawn between the aim of monitoring wild populations using scent analysis and the aims of the "electronic nose", first proposed by Persaud and Dodd (1982). The term "electronic nose" encompasses many instruments that have been developed to mimic human noses in the way they detect odours or flavours using pattern recognition to detect complete scent signals rather than separation techniques such as gas chromatography. The main applications of the "electronic nose" identified by Röck et al. (2008) were the food and beverage industry, environmental monitoring and disease diagnosis and state that it is not adapted to mammalian scent. The variety of data acquisition (feature selection, scaling and normalisation) and analysis (pattern recognition may be model based, supervised or unsupervised) methods are, however, similar, and here too there are no general guidelines to determine the appropriate strategy. Despite this, commonalities do exist, for example, a multivariate approach (as employed in this thesis) is clearly preferable and unlikely to produce false positives.

7.2 Chemical differences in otter scent

It was only possible to sample a large number of individuals by using scent samples originating from otter carcasses, but each sample represents a single time point in that otter's life. Multiple samples can be collected over time from the same individual otters in captivity, but the number of individuals sampled is restricted. Both sample types have the advantage of known origin and by combining analyses of the two I was able to examine multiple sources of variation in otter scent. In common with much of the previous work on mustelid scent communication (see review table in Chapter 1), categorical differences in otter scent relating to age, sex and reproductive status were

found in Chapter 4, between groups housed together in Chapter 5 and between genetic subpopulations in Chapter 6. Differences relating to individual identity and female hormonal state were found in Chapter 5. Chapter 3 describes variation in otter scent between sample types (faeces or anal gland secretion) and as a result of decay. Surprisingly, it appears diet does not influence otter scent (Chapter 3). Clearly many variables explain variation in otter scent as has been detailed for other well studied species, for example badgers (Buesching et al., 2002a, b, c).

These findings can be used to suggest communicative functions of spraint. Differences in scent relating to otter age, sex, reproductive status and female reproductive hormone levels suggest these signals are under hormonal control and may be used in mate attraction. The communication of individual identity could support either the mate attraction (Kruuk, 2006), scent matching (Gosling, 1982) or resource use signalling (Kruuk, 1992) hypotheses, although not entirely necessary for the later. There is recent evidence to support the latter hypothesis from the distribution of spraints (Prenda and Granado-Lorencio, 1996; Remonti et al., 2011), but given the lack of evidence for signalling prey type (Chapter 3) this may reflect the location of resource but not the type of resource. Given the geographical distances between the organisations that spraints were obtained from to investigate individual identity and the solitary nature of otters, it is likely that group differences found in Chapter 5 are similar to the genetic subpopulation differences found in Chapter 6. The communication of genetic relatedness through scent has been suggested to function in kin recognition and inbreeding avoidance or nepotism (Charpentier et al., 2008) and phenotype matching (Sun and Müller-Schwarze, 1998b). The genetic subpopulations in the otter populations of England and Wales are thought to be as a result of population declines and repopulation by small isolated populations (Hobbs, 2011) and differences in scent reflect this.

Several Experiments in this study each explained a small amount of variation in otter scent. In Chapter 4, analysis of scent allowed 79% of adult female samples (excluding pregnant and lactating) to be correctly identified. The finding of differences in scent correlated to progesterone level (Chapter 5) may explain why this classification was not

100%. Similar differences may exist for males, explaining the even lower correct classification for them (70%). For example weaker, less competitive males have lower testosterone and there is evidence of scent differences relating to dominance status, competition or testosterone concentration in many species (e.g. humans (Adolph et al., 2010; Havlicek et al., 2005), monkeys (Setchell et al., 2010), rodents (Liu et al., 2010; Zhang et al., 2001), deer (Gassett et al., 1996)). Given this relationship between testosterone and scent, the scent of males with lower testosterone may be more similar to that of females. The research in Chapter 5 is the first attempt to combine measurements of female reproductive hormones with scent analysis for any species. Previous research has linked categorical stages of the reproductive cycle with scent and so has not been able to demonstrate the continuous correlation between progesterone concentrations and scent that is demonstrated here for otters.

7.3. Can chemical information be used to identify otters from spraint?

The aim of this research is to develop a technique to identify otters through scent analysis of spraint, which could be used to non-invasively research wild populations in more detail than is currently possible through spraint distribution surveys. Many of the variables investigated, including age, sex, reproductive status, progesterone level, location and subpopulation, explained a small amount of variation in otter scent. There may be additional sources of variation that time and resources did not allow investigation of in the scope of this thesis, for example health status, impact of substrate and weather conditions. Given the large number of variables that explain variation in otter scent, it is not currently possible to control for all and therefore predict otter identity from samples of unknown origin. This is not exclusive to otter scent. Despite the 25 years of research since Persuad and Dodd (1982) first proposed the "electronic nose" it is still not possible to mimic the discriminatory ability of the mammalian olfactory system (Röck et al., 2008).

Key to the use of chemical analysis for monitoring purposes is answering the question; do otters have an individual scent signature? Although significant differences between individual otters were found in Chapter 5, the amount of variation that this explained was very small, meaning it could not reliably be used alone to identify otters.

Combining scent analysis with location information does not improve estimations of otter numbers and ranges as samples originating from different individuals may be located close together. Other variables such as age and sex could be used to examine differences in habitat use by different types of otters. As sex differences were the result of complex VOC mixtures it is not possible to identify sex easily. Age differences on the other hand were based on the relative abundance of two compounds and with validation on spraints from captive juveniles this may provide a way to estimate the age of otters from spraint, an outcome which is not possible from DNA analysis. Although genetic monitoring offers a lot to the conservation and management of wildlife populations it is not without limitations. Genetic monitoring can't detect parameters that change within an animal's lifetime such as age, dominance and reproductive status, but odour analysis can. DNA analysis and odour analysis could therefore complement each other by combing the two techniques to gain more information from samples. DNA analysis is subject to genotyping errors (Schwartz et al., 2007), particularly for otter spraintwhere success is typically between 14% and 65% (Hájková et al., 2009). To improve genotyping success samples need to be fresh (Coxon et al., 1999; Hájková et al., 2009), whereas there is potential for VOCs to be recovered from older, desiccated samples by rewetting (Burnham et al., 2008; Paul, 2010). Thus, with further development, VOC analysis could still offer advantages over genetic monitoring. Furthermore, by selecting for "primary odour" (those constituents which are stable over time regardless of diet and environmental factors), a 99.54% success rate of VOC analysis in identifying human individuals has been reported (Curran et al., 2010). At the present time this has only been achieved for humans by comparison to known reference samples, which would not be possible from wildlife populations. This method could be applied to wildlife reintroductions where prior to release reference samples can be collected and analysed from captivity and after release samples collected in the wild could be analysed to identify individuals and monitor movements and reintroduction success.

Chemical analysis of scent mark VOCs through GCMS is expensive (minimum £18 per sample at Cardiff University Bioscience Analytical Unit) especially when compared to spraint distribution surveys. This cost would prohibit wide use, but it could still be used on a smaller scale as a non-invasive research tool to answer specific ecological

questions. Additionally, whilst conducting this research I have received requests from numerous sources interested in having a small number of samples analysed, for example ecological consultants wishing to know what types of otters are using an area which is being assessed for development.

7.4 Relevance of scent to otters

Systematic testing of the scent discrimination ability of any otter species is lacking, with only two published articles (Rostain et al., 2004; Rozhnov and Rogoschik, 1994). This is probably due to the low number of some otter species in captivity and the difficulty in maintaining some species in captivity. Chapter 2 contains the first experimental attempts to test if Eurasian otters discriminate between spraints from males and females, or with high and low progesterone. Improvements were also made on the design used by Rozhnov and Rogoschik (1994) to test the effect of spraint freshness. Results suggested that otters can discriminate between sex and hormonal state of the depositor by sniffing spraint and that the same interest is shown in fresh spraint and that aged by four days (no other lengths of aging were tested but may be possible in the future). The most significant finding of these experiments was that otters make direct contact with spraint, which is contrary to previous assumptions about Eurasian otter behaviour. This suggests that similar to other species, non-volatiles are involved in otter scent communication (Nevison et al., 2003).

In this thesis I employed behavioural experiments and chemical analysis. Combining the two disciplines provides much more powerful and valid investigations of scent communication (e.g. banded mongooses (Jordan et al., 2010) or spotted hyaena (Burgener et al., 2009)) than either technique alone. Without additional bioassays of behavioural responses of otters to scent it is not possible to surmise that these chemical findings are relevant to otters, however, they do provide suggestions about what may be communicated between individuals. Otters are generally solitary and with large home ranges, signals in otter scent (found in Chapters 4 and 5) may help to mediate many social interactions. Signals relating to sex and reproductive status (Chapter 2 and 4), age (Chapter 4) and individual identity (Chapter 5) may allow otters to locate potential mates and avoid antagonistic interactions such as infanticide (observed by Simpson and

Coxon, 2000) or with competitors for mates or resources. These differences in scent are not exclusive to solitary species though as they are also found in group living species (e.g. badgers, Buesching et al., 2002 b, c).

Chapter 2 provides evidence for otters learning the locations of scent. Many authors have described how otters deposit spraint in conspicuous locations, with the same site regularly used, often for many years (e.g. Erlinge, 1967; Chanin, 1985; Kruuk, 2006). The finding that otters learn the location of spraint sites suggests spraint is located by memorising the location rather than always by chance encounters with spraint. There is little published literature on any species about the learning of likely scent locations in the absence of actual scent as shown in Chapter 2 and so this is worthy of further investigation. The location of spraint sites are so conspicuous that they are used by field ecologists to detect and monitor the presence of otters. There has been some question, however, as to whether these represent all spraints deposited by otters (Jenkins and Burrows, 1980; Ostman et al., 1985; Kruuk, 2006). If remaining undetected is an advantage for an individual, for example, a female with very young cubs, then spraints may be deposited in less regular locations, for example in water.

7.5 Limitations and future research

The unavoidable small sample size for the behavioural experiments and testing for an individual scent signature limit the conclusions from these experiments. Due to their solitary and nocturnal nature Eurasian otters are not commonly kept by zoos and so samples from known animals are only available from a small number of animal rescue centres. Other species regularly kept in captivity (e.g. mice, lemurs, ferrets, mink) make better models for investigating scent communication but the species-specific nature of scent communication in terms of animal biology and behavioural ecology, limit generalisations of findings from model species. Additional behavioural experiments would be useful to expand the sample size, and test other parameters, for example, individual recognition and the age of the otter. It would be interesting to test whether the single compounds found to differentiate between juvenile and adult otters (indole and 2 pentyl furan) are detected by otters. Furthermore there is some evidence that the

hormonal status of the receiver can impact what is detected (Woodley and Baum, 2003) and this should be controlled for in future experiments.

In Chapter 2 otters did not discriminate between fresh samples and a section of the same sample aged for four days. It would be interesting to examine for how long this is maintained. At Cardiff University we have started to investigate the effect of aging and different weather conditions on the longevity of spraint scent. Initial analysis suggests scent is conserved for longer in wet and cold conditions (Paul, 2010). Further investigation is needed to determine how long individual, age and sex differences are maintained. This is an important step if scent analysis is to be used to determine otter identity from spraint collected in the wild. Modelling of the scent differences found in this thesis would also be required to enable prediction from the scent of unknown samples.

More work is needed to investigate the frequency of the oestrous cycle of female otters. Measuring faecal hormone metabolites of females over a longer time period and in different social situations, in particular before and after an introduction of an unfamiliar male may allow description of the reproductive cycle. Otter scent is correlated with progesterone (Chapter 5), further investigation could determine if this relationship is maintained with changes in oestrogen concentrations.

Behavioural observations (Chapter 2) provided evidence that otters may gather information from non-volatile components of spraints as well as volatiles. Further research should be undertaken to establish the function of these two components. In mice, the ownership signal is involatile (Nevison et al., 2003) and associations are learned between volatiles and non-volatiles (Hurst, 2009). The same may not be true for carnivores but there has been little investigation of non-volatile scent communication in species other than mice. At Cardiff University Otter Project we have started to collaborate with Jane Hurst and Rob Beynon at Liverpool University to investigate differences in proteins in otter scent gland secretions. This may provide age, sex and individual differences that are easier to interpret than those found in VOCs in this thesis

and therefore be more useful for use as non-invasive monitoring of wild otter populations.

If the differences in the colour of anal gland secretions between adult and juvenile otters found in Chapter 4 are also true of spraints, this could offer additional information to surveys. In otter surveys, the colour of spraints is currently used only partially; to distinguish between old and fresh spraints (older spraints typically being greyer). Chapter 4 highlighted a difference in human perception of the smell of adult and juvenile otter anal gland secretions; juvenile samples were unpleasant smelling. This has not been recorded before and is potentially worrying as field ecologists frequently distinguish between otter spraints and the scat of other carnivores based on smell. It is possible that juvenile otter spraints could be mistaken for scat of American mink, an invasive species in the UK. As development decisions are based on these observations of scat in the field, the sprainting behaviour of juvenile otters and the frequency of people misidentifying spraints, should be investigated as a matter of urgency.

An emerging field of semiochemistry and ecotoxicology research is the effect of pollution on chemical communication. Pollution has been reported to disrupt the chemical communication between fish, in particular species recognition (Fisher et al., 2010) and shoaling behaviour (Ward et al., 2008). Impacts may be on the animal that deposits the scent signal or on how animals perceive the signal (Lürling and Scheffer, 2007). The complexity of relevant chemicals, receptors and reactions means much more research is required to develop testing systems (Klaschka, 2009). The impact of this so-called "info-disruption" may have far reaching consequences for ecosystem functioning and conservation management (Lürling and Scheffer, 2007). This is disturbing; particularly with regards to a species such as the Eurasian otter that relies on scent communication for the majority of social interactions and that was previously nearly driven to extinction due to pollution. Environmental policy makers should take heed of these published warnings from other species and invest in the monitoring of emerging pollutants and their impacts on scent communication.

7.6 Conclusion

The inherent complexity of scent signalling in otters reported here mean that it is not possible, on the basis of current research, to reliably predict otter identity through scent analysis of spraint samples. This thesis does, however, make several major steps towards unravelling the complexity of otter scent and presents findings which are novel to the field of semiochemistry; the links between scent and progesterone levels, and genetic subpopulations in the wild. This study also adds to the knowledge of otter biology and behaviour.

References

Adolph, D., Schlösser, S., Hawighorst, M. and Pause, B.M. 2010. Chemosensory signals of competition increase the skin conductance response in humans. *Physiology & Behavior*, 101, 666–671.

Albone, E. S. 1984. *Mammalian Semiochemistry: The Investigation of Chemical Signals between Mammals*. New York: Wiley.

Amstislavsky, S. and Ternovskaya, Y. 2000. Reproduction in mustelids. *Animal Reproduction Science*, 60, 571-581.

Anderson, H.M., McCafferty, D.J., Saccheri, I.J. and McCluskie, A.E. 2006. Non-invasive genetic sampling of the Eurasian otter (*Lutra lutra*) using hairs. *Hystrix*, 17(1), 65-77.

Anderson, M.J. 2001. A new method for non-parametric multivariate analysis of variance. *Austral Ecology*, 26, 32–46.

Andreolini, F., Jemiolo, B. and Novotny, M. 1987. Dynamics of excretion of urinary chemosignals in the house mouse (*Mus musculus*) during the natural estrous cycle. *Experientia*, 43, 998-1002.

Bagley, K.R., Goodwin, T.E., Rasmussen, L.E.L. and Schulte, B.A. 2006. Male African elephants, *Loxodonta africa*, can distinguish oestrous status via urinary signals. *Animal Behaviour*, 71, 1439-1445.

Bateman, H.L., Bond, J.B., Campbell, M., Barrie, M., Riggs, G., Snyder, B. and Swanson, W.F. 2009. Characterization of basal seminal traits and reproductive endocrine profiles in North American river otters and Asian small-clawed otters. *Zoo Biology*, 28 (2), 107-126.

Berzins, R. and Helder, R. 2008. Olfactory communication and the importance of different odour sources in the ferret (*Mustela putorius f. furo*). *Mammalian Biology*, 73 (5), 379-387.

Boulet M., Charpentier, M.J.E. and Drea, C.M. 2009. Decoding an olfactory mechanism of kin recognition and inbreeding avoidance in a primate. *BMC Evolutionary Biology*, 9:281.

Bradshaw, A., Beckmann, M., Stevens, R. and Slater, F. 2001. Anal scent gland secretion of the European Otter (*Lutra lutra*). In *Chemical Signals in Vertebrates* 9. New York: Kluwer Academic/Plenum Publishers, pp. 313-319.

Brennan, P.A. and Kendrick, K.M. 2006. Mammalian social odours: Attraction and individual recognition. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 361, 2061-2078.

Brinck, C., Erlinge, S. and Sandell, M. 1983. Anal sac secretion in mustelids - a comparison. *Journal of Chemical Ecology*, 9, 727-745.

Brinck, C., Gerell, R. and Odham, G. 1978. Anal pouch secretion in mink *Mustela vison*. *Oikos*, 30, 68-75.

Broekhuizen, S., Lammertsma. D.R. and Müskens, G.J.D.M. 2007. Re-mating in otter (*Lutra lutra*). *Lutra* 50 (2), 135-139.

Broquet, T., Menard N. and Petit, E. 2007. Noninvasive population genetics: a review of sample source, diet, fragment length and microsatellite motif effects on amplification success and genotyping error rates. *Conservation Genetics*, 8 (1), 249-260.

Brown, R.E. 1979. Mammalian social odors: A critical review. *Advances in the Study of Behavior*, 10, 103-162.

Brown, R.E. and McDonald, D.W. 1985. *Social Odours in Mammals* Vols 1& 2. Oxford: Claredon.

Buesching, C.D., Newman, C. and Macdonald, D.W. 2002a. Variations in colour and volume of the subcaudal gland secretion of badgers (*Meles meles*) in relation to sex, season and individual-specific parameters. *Mammalian Biology*, 67 (3), 147-156.

Buesching, C.D., Waterhouse, J.S. and Macdonald D.W. 2002b. Gas-Chromatographic analyses of the subcaudal gland secretion of the european badger (*Meles meles*) Part I: chemical differences related to individual parameters. *Journal of Chemical Ecology*, 28, 41-56.

Buesching, C.D., Waterhouse, J.S. and Macdonald D.W. 2002c. Gas-Chromatographic analyses of the subcaudal gland secretion of the European badger (*Meles meles*) part II: time-related variation in the individual-specific composition. *Journal of Chemical Ecology*, 28, 57-69.

Buesching, C.D., Stopka, P. and MacDonald, D.W. 2003. The social function of allo-marking in the European badger (*Meles meles*). *Behaviour*, 140, 965-980.

Burger, B.V. 2005. Mammalian semiochemicals. *Topics in Current Chemistry*, 240, 231-278.

Burnham, E., Bender, L.C., Eiceman, G.A., Pierce, K.M. and Prasad, S. 2008. Use of volatile organic components in scat to identify canid species. *Journal of Wildlife Management*, 72, 792-797.

Burgener, N., Dehnard, M., Hofer, H. and East, M.L. 2009. Does anal scent signal identity in the spotted hyaena? *Animal Behaviour*, 77, 707-715

Burger, B.V., Viviers, M. Z., Bekker, J. P. I., le Roux, M., Fish, N., Fourie, W. B. and Weibchen, G. 2008. Chemical characterization of territorial marking fluid of male Bengal tiger, *Panthera tigris*. *Journal of Chemical Ecology*, 34, 659-671.

Campbell-Palmer, R. and Rosell, F. 2011. The importance of chemical communication studies to mammalian conservation biology: A review. *Biological Conservation*, 144, 1919-1930.

Carss, D.N. and Parkinson, S.G. 1996. Errors associated with otter *Lutra lutra* faecal analysis. I. Assessing general diet from spraints. *Journal of Zoology*, 238(2), 301-317.

Chadwick, E.A. 2007. *Post mortem study of otters in England and Wales 1992-2003*. Environment Agency Science Report. SC010065/SR.

Chadwick, E.A. and Sherrard Smith, E. 2010. Pregnancy among otters (*Lutra lutra*) found dead in England and Wales: foetal development and lack of seasonality. *IUCN Otter Specialist Bulletin*, 27(1), 33-41.

Chanin, P. 1985. *The Natural History of Otters*. London: Christopher Helm.

Chanin, P. 2003. *Ecology of the European Otter*. Conserving Natura 2000 Rivers Ecology Series No. 10. Peterborough: English Nature.

Chanin, P. 2005. *Otter surveillance in SACs: testing the protocol.* English Nature Research Report Number 664. Peterborough: English Nature.

Cheetham, S.A., Thom, M.D., Jury, F., Ollier, W.E.R., Beynon, R.J. and Hurst, J.L. 2007. The genetic basis of individual-recognition signals in the mouse. *Current Biology*, 17, 1771–1777.

Charpentier, M.J.E., Boulet, M. and Drea, C.M. 2008. Smelling right: The scent of male lemurs advertises genetic quality and relatedness. *Molecular Ecology*, 17, 3225-3233.

Clarke, P.M.R., Barrett, L. and Henzi, S.P. 2009. What role do olfactory cues play in chacma baboon mating? *American Journal of Primatology*, 71 (6), 493-502.

Cloe, A.L. Woodley, S.K. Waters, P. Zhou, H. and Baum M.J. 2004. Contribution of anal scent gland and urinary odorants to mate recognition in the ferret. *Physiology & Behavior* 82, 871–875.

Conroy, J.W.H., Watt, J., Webb, J.B., and Jones, A. 2005. *A Guide to the Identification of Prey Remains in Otter Spraint 3rd edition.* London: Mammal Society.

Coxon, K., Chanin, P., Dallas, J. and Sykes, T. 1999. *The use of DNA fingerprinting to study the population dynamics of otters (*Lutra lutra*) in southern Britain a feasibility study*. Environment Agency R&D Project W1-025.

Crawford, A. 2010. *Fifth otter survey of England 2009-2010*. Bristol: Environment Agency.

Crawford, A., Evans, D., Jones, A. and McNulty, J. 1979. *Otter survey of Wales 1977-78.* Society for the Promotion of Nature Conservation, Nettleham.

Crump, D.R. 1980a. Anal gland secretion of the ferret (*Mustela putorius forma furo*). *Journal of Chemical Ecology*, 6, 837-844.

Crump, D. R. 1980b. Thietanes and dithiolanes from the anal gland of the stoat (*Mustela erminea*). *Journal of Chemical Ecology*, 6, 341-347.

Curran, A.M. Prada, P.A. and Furton, K.G. 2010. The differentiation of the volatile organic signatures of individuals through SPME-GC/MS of characteristic human scent compounds. *Journal of Forensic Sciences*, 55(1), 50-57.

Dallas, J.F., Coxon, K.E., Sykes, T., Chanin, P.R.F., Marshall, F., Carss, D.N., Bacon, P.J., Piertney, S.B. and Racey, P.A. 2003. Similar estimates of population genetic

composition and sex ratio derived from carcasses and faeces of Eurasian otter *Lutra lutra*. *Molecular Ecology*, 12, 275-282.

Davies, J.M., Lachno, D.R. and Roper, T.J. 1988. The anal gland secretion of the European badger (*Meles-Meles*) and its role in social communication. *Journal of Zoology*, 216, 455-463.

Davies, M.J. 2008. *Chemical communication in the European otter, Lutra lutra*. Unpublished doctoral thesis, University of Hull.

Davis, J.A. 1978. A classification of otters. In: Duplaix, N. (eds.). *Otters: Proceedings of the First Meeting of the Otter Specialist Group.* Morges: IUCN, pp 14-33.

Decker, D.M., Ringelberg, D. and White, D.C. 1992. Lipid components in anal scent sacs of 3 mongoose species (*Helogale-Parvula, Crossarchus-Obscurus*, *Suricata-Suricatta*). *Journal of Chemical Ecology*, 18, 1511-1524.

del Barco-Trillo, J., LaVenture, A.B. and Johnston, R.E. 2009. Male hamsters discriminate estrous state from vaginal secretions and individuals from flank marks. Behavioural Processes, 82 (1), 18-24.

de Monte, M. and Roeder, J.J. 1990. Responses to interspecific and intraspecific scent marks in pine martens *Martes martes. Journal of Chemical Ecology*, 16, 611-618.

Dixon S.J., Brereton R.G., Soini H.A., Novotny M.V. and Penn D.J. 2006. An automated method for peak detection and matching in large gas chromatography-mass spectrometry data sets. *Journal of Chemometrics*, 20, 325-340.

Duplaix, N. 1980. Observations on the ecology and behaviour of the giant river otter *Pteronura brasiliensis* in Suriname. *Revue d'Ecologie (Terre Vie)* 34,495–520.

Durbin, L. 1989. Some responses of otters *Lutra lutra* to strange and familiar spraints. *Lutra*, 32, 132-138.

Durbin, L.S. 1998. Habitat selection by five otters *Lutra lutra* in rivers of northern Scotland. *Journal of Zoology*. 245, 85-92.

Ebert, C., Knauer, F., Storch, I. and Hohmann, U. 2010. Individual heterogeneity as a pitfall in population estimates based on non-invasive genetic sampling: a review and recommendations. *Wildlife biology*, 16 (3), 225 -240.

Ebling, F. J. 1977. Hormonal control of mammalian skin glands. In: Muller-Schwarze, D and Mozell, M.M. (ed.) *Chemical Signals in Vertebrates*. New York: Plenum Press.

Erlinge, S. 1967. Home range of the otter *Lutra lutra L.* in southern Sweden. *Oikos*, 18, 186–209.

Erlinge, S. 1968. Territoriality of the otter *Lutra lutra L. Oikos*, 19, 81-98.

Erlinge, S., Sandell, M. and Brinck, C. 1982. Scent-marking and its territorial significance in stoats, *Mustela erminea*. *Animal Behaviour*, 30, 811-818.

Eisenberg, J.F. and Kleiman, D.G. 1972. Olfactory communication in mammals. *The Annual Review of Ecology, Evolution, and Systematics*, 3, 1-32.

Fasano, R. and Milone, M. 1993. Lipidic composition of otter's (*Lutra lutra*) spraints on the middle watercourse of the Sele river (Southern Italy). In *Italian Symposium on Carnivores. Pavia, October, 1993.*

Fisher, H.S., Wong, B.B.M. and Rosenthal, G.G. 2010. Alteration of the chemical environment disrupts communication in a freshwater fish. *Proceedings of the Royal Society B*, 273, 1187-1193.

Garcia De Leaniz, C., Forman, D.W., Davies, S. and Thomson, A. 2006. Non-intrusive monitoring of otters (*Lutra lutra*) using infrared technology. *Journal of Zoology*, 270(4), 577 584.

Garner, C.E., Smith, S., de Lacy Costello, B., White, P., Spencer, R., Probert, C.S.J. and Ratcliffe, N.M. 2007. Volatile organic compounds from feces and their potential for diagnosis of gastrointestinal disease. *The Journal of the Federation of American Societies for Experimental Biology*, 21, 1675-1688.

Gassett, J.W., Wiesler, D.P., Baker, A.G., Osborn, D.A. and Miller, K.V., Marchinton, R.L. and Novotny, M. 1996. Volatile compounds from interdigital gland of male white-tailed deer *(Odocoileus virginianus). Journal of Chemical Ecology*, 22(9), 1689- 1696.

Gerling, S. and Yahr, P. 1982. Maternal and paternal pheromones in gerbils. *Physiology and Behavior*, 28, 667-673.

Giaquinto, P. C., da Silva Berbert, C. M. and Delicio, H.C. 2010. Female preferences based on male nutritional chemical traits. *Behavioral Ecology and Sociobiology*, 64, 1029-1035.

Goodrich, B.S. and Mykytowy, R. 1972. Individual and sex differences in chemical composition of pheromone-like substances from skin glands of rabbit, *Oryctolagus-Cuniculus*. *Journal of Mammalogy*, 53, 540-548.

Gorman, M. L., Jenkins, D. and Harper, R J. 1978. Anal scent sacs of otter (*Lutra lutra*). *Journal of Zoology*, 186, 463-474.

Gorman, M.L., Kruuk, H. and Leitch, A. 1984. Social functions of the sub-caudal scent gland secretion of the European badger *Meles meles* (Carnivora: Mustelidae). *Journal of Zoology*, 204, 549–559.

Gosling L M. 1982. A reassessment of the function of scent marking in territories. *Zeitschrif für Tierpsychologie*. 60(2), 89-118.

Green, R. 2000. Sexual differences in the behaviour of young otters (*Lutra lutra*). *IUCN Otter Specialist Group Bulletin*, 17, 20-30.

Green, J., Green, R. and Jefferies, D.J. 1984. A radio-tracking survey of otters *Lutra lutra* on a Perthshire river system. *Lutra*, 27, 85-145.

Guter, A., Dolev, A., Saltz, D. and Kronfeld-Schor, N. 2008. Using videotaping to validate the use of spraints as an index of Eurasian otter (*Lutra lutra*) activity. *Ecological Indicators*, 8, 462-465.

Hagey, L. and MacDonald, E. 2003. Chemical cues identify gender and individuality in Giant pandas (*Ailuropoda melanoleuca*). *Journal of Chemical Ecology*, 29, 1479-1488.

Hájková, P., Zemanová, B., Roche, K. and Hájek, B. 2009. An evaluation of field and noninvasive genetic methods for estimating Eurasian otter population size. *Conservation Genetics*, 10, 1667-1681.

Halpin, Z.T. 1986. Individual odors among mammals: origins and functions. *Advances in the Study of Behavior*, 16, 39-70.

Harrington, L.A., Harrington, A.L. and MacDonald, D.W. 2009. The smell of new competitors: The response of american mink, *Mustela vison*, to the odours of otter, *Lutra lutra* and polecat, *M. putorius*. *Ethology*, 115, 421-428.

Harrington, L.A., Harrington, A.L., Hughes, J., Stirling D. and Macdonald, D.W. 2010. The accuracy of scat identification in distribution surveys: American mink, *Neovison vison*, in the northern highlands of Scotland. *European Journal of Wildlife Research*, 56, 377-384.

Harris, S. and Yalden, D.W., editors. 2008. *Mammals of the British Isles: Handbook*, 4th Edition. Southampton: The Mammal Society.

Hauer, S., Ansorge, H. and Zinke, O. 2002. Reproductive performance of otters *Lutra lutra* (Linnaeus, 1758) in Eastern Germany: low reproduction in a longterm strategy. *Biological Journal of the Linnaean Society*, 77, 329-340.

Hawken, P.A.R. and Beard, A.P. 2009. Ram novelty and the duration of ram exposure effects the distribution of mating in ewes exposed to rams during the transition into the breeding season. *Animal Reproduction Science*, 111(2–4), 249–260.

Havlicek, J., Roberts, S.C. and Flegr, J. 2005. Women's preference for dominant male odour: effects of menstrual cycle and relationship status. *Biology Letters*, 1, 256–259.

Hayes, R.A., Richardson, B.J., Claus, S.C. and Wyllie, S.G. 2002. Semiochemicals and social signaling in the wild European rabbit in Australia: II. Variations in chemical composition of chin gland secretion across sampling sites. *Journal of Chemical Ecology*, 28, 2613-2625.

Heath, R.R. and Dueben, B.D. 1998. Analytical and preparative gas chromatography. In Millar, J.G. and Haynes, K.F. eds. *Methods in Chemical Ecology*. Norwell, Massachusetts, USA: Kluwer Academic Publishers.

Heggberget, T.M. and Christensen, H. 1994. Reproductive timing in Eurasian otters on the coast of Norway. *Ecography*, 17,339–348.

Herr, J., Müller-Schwarze, D. and Rosell F. 2006. Resident beavers (*Castor canadensis*) do not discriminate between castoreum scent marks from simulated adult and subadult male intruders. *Canadian Journal of Zoology*, 84, 615-622.

Hobbs, G.I., Chadwick, E.A., Bruford, M.W. and Slater, F.M. 2011. Bayesian clustering techniques and progressive partitioning to identify population structuring within a recovering otter population in the UK. *Journal of Applied Ecology*, 48, 1206-1217.

Hobbs, N.J., Aven, A.M. and Ferkin M.H. 2008. Self-grooming response of meadow voles to the odor of opposite-sex conspecifics in relation to the dietary protein content of both xexes. *Ethology*, 114, 1210-1217.

Hobbs, N.J., and Ferkin, M.H. 2011. Dietary protein content affects the response of meadow voles, *Microtus pennsylvanicus*, to over-marks. *Acta Ethologica*. 14(2), 57-64.

Huck, U.W., Bracken, A.C. and Lisk, R.D. 1983. Female-induced pregnancy block in the golden hamster. *Behavioral and Neural Biology*, 38, 190–193.

Huck, U.W., Lisk, R.D. and McKay, M.V. 1988a. Social dominance and reproductive success in pregnant and lactating golden hamsters (*Mesocricetus auratus*) under seminatural conditions. *Physiology and Behavior*, 44, 313–319.

Huck, U.W., Lisk, R.D., Miller, K.S. and Bethel. A. 1988b. Progesterone levels and socially-induced implantation failure and fetal resorption in golden hamsters (*Mesocricetus auratus*). *Physiology and Behavior*, 44, 321–326.

Hurlbert, S.H. 1984. Pseudoreplication and the design of ecological field experiments. *Ecological Monographs*, 54, 187-211.

Humphries, R.E., Robertson, D.H.L., Beynon, R.J. and Hurst, J.L. 1999. Unravelling the chemical basis of competitive scent marking in house mice. *Animal Behaviour*. 58, 1177–1190.

Hung, C-M., Li, S-H. and Lee, L-L. 2004. Faecal DNA typing to determine the abundance and spatial organisation of otters (*Lutra lutra*) along two stream systems in Kinmen. *Animal Conservation*, 7(3), 301-311.

Hurst, J. L. 1989. The complex network of olfactory communication in populations of wild house mice *Mus domesticus* rutty: urine marking and investigation within family groups. *Animal Behaviour*. 37, 705–725.

Hurst, J. L. 2009. Female recognition and assessment of males through scent. *Behavioural Brain Research*. 200(2), 295-303.

Hurst, J.L. and Beynon, R.J. 2010. Making progress in genetic kin recognition among vertebrates. *Journal of Biology*, 9:13.

Hurst, J.L., Payne, C.E., Nevison, C.M., Marie, A.D., Humphries, R.E., Robertson, D.H.L., Cavaggioni, A. and Beynon, R.J. 2001. Individual recognition in mice mediated by major urinary proteins. *Nature*, 414, 631-634.

Hutchings, M.R. and White, P.C.L. 2000. Mustelid scent-marking in managed ecosystems: implications for population management. *Mammal Review*, 30 (3-4), 157-169.

Janotová, K. and Stopka, P. 2009. Mechanisms of chemical communication: The role of major urinary proteins. *Folia Zoologica*, 58, 41-55.

Jefferies, D.J. 1966. The value of otter *Lutra lutra* surveying using spraints: an analysis of its successes and problems in Britain. *Journal of the Otter Trust*, 1, 25-32

Jefferies, D.J. and Hanson, H.M. 2000. The role of dieldrin in the decline of the otter (*Lutra lutra*) in Britain: the analytical data. In Conroy JWH Yoxon P and Gutleb AC (Eds), *Proceedings of the First Otter Toxicology Conference, Skye, Sept 2000* (pp.95-143). International Otter Survival Fund, Broadford, Scotland.

Jenkins, D. and Burrows G.O. 1980. Ecology of otters in northern Scotland .III. The use of faeces as indicators of otter (*Lutra lutra*) density and distribution. *Journal of Animal Ecology*, 49, 755-774.

Jenkins, D., Makepeace, M. and Gorman, M.L. 1981. Two dimensional electrophoresis of otter (*Lutra lutra*) anal gland secretions. *Journal of the Zoological Society*, 195, 558-559.

JNCC. 2007. *UK BAP Priority Species pages Lutra lutra-Version2*. Joint Nature Conservation Committee.

Johansson, B.G. and Jones, T.M. 2007. The role of chemical communication in mate choice. *Biological Reviews*, 82, 265-289.

Johnston, R.E., Chiang, G. and Tung, C. 1994. The information in scent over-marks of golden hamsters. *Animal Behaviour*, 48(2), 323-330.

Jordan, N.R., Manser, M.B., Mwanguhya, F., Kyabulima, S., Rüedi, P. and Cant, M.A. 2010 Scent marking in wild banded mongooses: 1. Sex-specific scents and overmarking. *Animal Behaviour*, 81(1), 31-42.

Jorga, W., Tschirch, W. and Eichhorn, S. 2004.Bemerkungen zur Tragezeit des Eurasischen Fischotters (*Lutra lutra*). *Beiträge zur Jagd- und Wildforschung*, 29, 297-406.

Kalinova, B., Podskalska, H., Ruzicka, J. and Hoskovec, M. 2009. Irresistible bouquet of death - or how are burying beetles (Coleoptera: Silphidae: Nicrophorus) attracted by carcasses. *Naturwissenschaften*. 96(8), 889-899.

Kalz, B., Jewgenow, K. and Fickel, J. 2006. Structure of an otter (*Lutra lutra*) population in Germany - results of DNA and hormone analyses from faecal samples. *Mammalian Biology*, 71, 321-335.

Kean, E.F. 2007. *Otter Video Monitoring Project*. Cheshire Wildlife Trust report

Kean, E.F., Muller, C.T. and Chadwick, E.A. 2011. Otter scent signals age, sex and reproductive status. *Chemical Senses* 36(6), 555-564.

Klaschka, U. 2009. A new challenge-development of test systems for the infochemical effect. *Environmental Science and Pollution Research*, 16 (4), 370-388.

Kleiman, D. 1966. Scent marking in the Canidae. *Symposia of the Zoological Society of London*, 18, 167-177.

Kleiman, D.G., Thompson, K.V. and Kirk Baer, C. 2010. *Wild Mammals In Captivity: Principles and Techniques for Zoo Management* (2nd edition). Chicago: The University of Chicago Press.

Kohn, M.H. and Wayne, R.K. 1997. Facts from feces revisited. *Trends in Ecology & Evolution*, 12, 223-227.

Koodsma, D.E., Byers, B.E., Goodale, E., Johnson, S., and Liu, W-C. (2001). Pseudoreplication in playback experiments, revisited a decade later. *Animal Behaviour*, 61, 1029-1033.

Kruuk H. 1992. Scent Marking by Otters (*Lutra lutra*) - Signalling the use of resources. *Behavioral Ecology*. 3(2), 133-140.

Kruuk, H. 1995. *Wild Otters: Predation and Populations.* Oxford: Oxford University Press.

Kruuk, H. 2006. *Otters Ecology, Behaviour and Conservation.* Oxford: Oxford University Press.

Kruuk, H. and Conroy, J.W.H. 1987. Surveying otter *Lutra lutra* populations: a discussion of problems with spraints. *Biological Conservation*, 41, 179 – 183.

Kruuk, H. and Moorhouse, A. 1991. The spatial organisation of otters (*Lutra lutra*) in Shetland. *Journal of Zoology*, London 224, 41-57.

Kruuk, H., Conroy, J.W.H., Glimmerveen U. and Ouwerkerk, E.J. 1986. The use of spraints to survey populations of otters *Lutra lutra*. *Biological Conservation* 35 187-194.

Kumar, K.R., Archunan, G., Jeyaraman, R. and Narasimhan, S. 2000. Chemical characterization of bovine urine with special reference to oestrus. *Veterinary Research Communications*, 24, 445-454.

Kwak, J., Willse, A., Matsumura1, K., M., Curran Opiekun, Yi, W., Preti, G., Yamazaki, K. and Beauchamp, G.K. 2008. Genetically-based olfactory signatures persist despite dietary variation. *PloS ONE*, 3(10), e3591.

Laengle, T., and Jorga, W. 2003. Studies on optimized enclosures for the European otter (*Lutra lutra*). *Zoologische Garten.* 73(1), 11-32.

Laidler, L. 1982. *Otters in Britain*. Newton Abbot: David & Charles.

Larson, S., Casson, C. J. and Wasser, S. 2003. Noninvasive reproductive steroid hormone estimates from fecal samples of captive female sea otters (*Enhydra lutris).* *General and Comparative Endocrinology*, 134(1), 18-25.

Lenton, E.J., Chanin, P.R.F. and Jefferies, D.J., 1980. *Otter Survey of England 1977-79.* London: Nature Conservancy Council.

Leuchtenberger C. and Mourão G. 2009. Scent-marking of giant otter in the Southern Pantanal, Brazil. *Ethology*, 115, 210-216.

Lévy, F., Keller, M. and Poindron, P. 2004. Olfactory regulation of maternal behavior in mammals. *Hormones and Behavior*, 46, 284-302.

Lewis, R.J., editor. 2007. *Hawley's Condensed Chemical Dictionary*. 15th ed. New York: Van Nostrand Rheinhold Co.

Liu, Y-J., Wang, D-W., Sun, L., Zhang, J-H. and Zhang, J-X. 2010. Flank gland-secreted putative chemosignals pertaining to photoperiod, endocrine states, and sociosexual behavior in golden hamsters. *Current Zoology*, 56 (6), 800- 812.

Luo, M., Fee, M.S., and Katz1, L.C. 2003. Encoding pheromonal signals in the accessory olfactory bulb of behaving mice. *Science*, 299, 1196-1201.

Lürling, M. and Scheffer, M. 2007. Info-disruption: pollution and the transfer of chemical information between organisms. *Trends Ecology and Evolution*, 22, 374–379.

Ma, W. and Klemm, W.R. 1997. Variations of equine urinary volatile compounds during the oestrous cycle. *Veterinary Research Communications*, 21, 437-446.

Ma, W., Wiesler, D. and Novotny, M.V. 1999. Urinary volatile profiles of the deermouse (*Peromyscus maniculatus*) pertaining to gender and age. *Journal of Chemical Ecology*, 25(3), 417-431.

Macdonald S.M. and Mason C.F. 1987. Seasonal marking in an otter population. *Acta Theriologica*, 32, 449-462.

Machiels, D., Van Ruth, S.M., Posthumus, M.A. and Istasse, L. 2003. Gas chromatography-olfactometry analysis of the volatile compounds of two commercial Irish beef meats. *Talanta*. 60, 755-764.

Madsen A.B. and Gaardmand B. (2000). Otter *Lutra lutra* monitoring in Denmark based on spraint surveys, collected carcasses and reported observations. *Lutra*, 43, 29-38.

Martin, G.B., Oldham, C.M., Cognié, Y. and Pearce, D.T.. 1986. The physiological response of anovulatory ewes to the introduction of rams—a review. *Livestock Production Science*, 15, 219–247.

Martineau, B. and Laflamme, D.P. 2002. Effect of diet on markers of intestinal health in dogs. *Research in Veterinary Science*, 72, 223-227.

Mason, C. F. and MacDonald, S. M. 1986. *Otters: Ecology and Conservation*. Cambridge: Cambridge University Press.

Massolo, A., Dani, F.R. and Bella N. 2009. Sexual and individual cues in the peri-anal gland secretum of crested porcupines (*Hystrix cristata). Mammalian Biology*, 74, 488-496.

Mason, C.F. and Macdonald, S.M. 1987. The use of spraints for surveying otter *Lutra lutra* populations - an evaluation. *Biological Conservation*, 41, 167-177.

Mason, C.F. and Macdonald, S.M. 1993. Impact of organochlorine pesticide-residues and PCBs on otters (*Lutra-Lutra*) - a study from western britain. *Science of the Total Environment*, 138 (1-3), 127-145.

Mathis, A. and Smith, R.J.F. 1993. Fathead minnows, *Pimephales-promelas*, learn to recognize Northern pike, *Esox-lucius*, as predators on the basis of chemical stimuli from minnows in the pikes diet. *Animal behaviour* 46(4), 645-656.

Melissen, A. 2000. *Husbandary Guidelines for Eurasian otter* Lutra lutra. EEP/Studbook. Leewarden, The Netherlands: Otterpark AQUALUTRA.

Meynell, P. 1999. *Otter semiochemistry: a proposal for the future conservation of* Lutra lutra. Honours dissertation, Cardiff University.

Miyazaki, M., Yamashita, T., Suzuki, Y., Saito, Y., Soeta, S., Taira, H. and Suzuki, A. 2006. A major urinary protein of the domestic cat regulates the production of felinine, a putative pheromone precursor. *Chemistry & Biology*, 13, 1071-1079.

Moestl, E., Messmann, S., Bagu, E., Robia, C. and Palme, R., 1999. Measurement of glucocorticoid metabolite concentrations in faeces of domestic livestock. *Journal of Veterinary Medicine Series A*, 46,621–631.

Müller-Schwarze D. 1971. Pheromones in black-tailed deer (*Odocoileus heminonus columbianus). Animal Behaviour*, 19, 141-152.

Müller-Schwarze, D. 1990. Leading them by their noses: Animal and plant odours for managing vertebrates. In: Macdonald, D.W., Müller-Schwarze, D. and Natynczuk, S.E. (eds.) *Chemical Signals in Vertebrates 5*. Oxford: Oxford University Press.

Müller-Schwarze, D. 2006. *Chemical Ecology of Vertebrates*. New York: Cambridge University Press.

Nelson, K. and Kruuk, H. 1997. The prey of otters: calorific content of eels (*Anguilla Anguilla*) and other fish, frogs (*Rana temporaria*) and toads (*Bufo bufo*). *IUCN Otter Specialist Bulletin*, 14, 75-80.

Nevison, C.M., Armstrong, S., Beynon, R.J., Humphries, R.E. and Hurst, J.L. 2003. The ownership signature in mouse scent marks is involatile. *Proceedings of the Royal Society B: Biological Sciences*, 270, 1957-1963.

Oksanen, J., Blanchet, F.G., Kindt, R. Legendre, P., Minchin, P.R., O'Hara, R.B., Simpson, G.L., Stevens, H.H. and Wagner, H. 2011. *vegan: Community Ecology Package.* R package version 2.0-0. http://CRAN.R-project.org/package=vegan

Osada, K., Yamazaki, K., Curran, M., Bard, J., Smith, B.P.C. and Beauchamp, G.K. 2003. The scent of age. *Philosophical Transactions of the Royal Society B,* 270, 929-933.

Ostman, J., Hillegaart, V. and Sandegren, F. 1985. Behavioural changes in captive female otters (*Lutra lutra*) around parturition. *Otters, Journal of the Otter Trust,* 1(8), 58-63.

Palphramand, K.L. and White, P.C.L. 2007. Badgers, *Meles meles,* discriminate between neighbour, alien and self scent. *Animal Behaviour,* 74, 429-436.

Pareja, M., Mohib, A., Birkett, M.A., Dufour, S. and Glinwood, R.T. 2009. Multivariate statistics coupled to generalized linear models reveal complex use of chemical cues by a parasitoid. *Animal Behaviour*, 77, 901-909.

Paul, S.P. 2010. *The lingering odour of otters; abiotic effects on the scent signals of Lutra lutra*. Honours dissertation, Cardiff University.

Penn, D.J., Oberzaucher, E., Grammer, K., Fischer, G., Soini, H.A., Wiesler, D., Novotny, M.V., Dixon, S.J., Xu, Y. and Brereton, R.G. 2007. Individual and gender fingerprints in human body odour. *Journal of the Royal Society Interface*, 4, 331-340.

Persaud, K.C. and Dodd, G. 1982. Analysis of discrimination mechanisms in the mammalian olfactory system using a model nose. *Nature*, 299, 352-355.

Podskalská, H., Růžička, J., Hoskovec, M. and Šálek, M. 2009. Use of infochemicals to attract carrion beetles into pitfall traps. *Entomologia Experimentalis et Applicata*, 132 (1), 59–64.

Polotti, P., Prigioni, C. and Fumagalli, R. 1995. Preliminary data on the ontogeny of some behavioural activities of captive otter cubs. *Hystrix*, 7(1-2), 279-284.

Porter, R.H. and Winberg, J. 1999. Unique salience of maternal breast odors for newborn infants. *Neuroscience and Biobehavioral Reviews*, 23, 439-449.

Prenda, J. and Granado-Lorencio, C. 1996. The relative influence of riparian habitat structure and fish availability on otter *Lutra lutra L.* sprainting activity in a small Mediterranean catchment. *Biological Conservation*, 76, 9–15.

Preston, J., Prodöhl, P., Portig A. and Montgomery, I. 2006. *Reassessing Otter Lutra lutra distribution in Northern Ireland.* Environment and Heritage Service Research and Development Series. No. 06/24.

Prigioni, C., Fumagalli, R., Schirru, L. and Carugati, C. 1995. Sprainting activity of captive otters: its relationship with breeding cycle and number of animals. *Hystrix*, 7, 297-301.

Prigioni, C., Remonti, L. and Balestrieri, A. 2006. Otter *Lutra lutra* movements assessed by genotyped spraints in southern Italy. *Hystrix*, 17(1), 91-96.

R Development Core Team. 2011. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org/.

Rajanarayanan, S. and Archunan, G. 2011. Identification of urinary sex pheromones in female buffaloes and their influence on bull reproductive behaviour. *Research in Veterinary Science*, 91(2), 301-5.

Rasmussen, L.E.L., Lee, T.D., Roelofs, W.L., Zhang, A. and Daves Jr, G.D. 1996. Insect pheromone in elephants. *Nature*, 379, 684.

Rasmussen, L.E.L., Lee, T.D., Zhang, A., Roelofs, W.L. and Daves Jr, G.D. 1997. Purification, identification, concentration and bioactivity of (Z)-7-Dodecen-1-yl acetate: Sex pheromone of the female Asian elephant, *Elephas maximus*. *Chemical Senses*, 22, 417-437.

Raymer, J., Wiesler, D., Novotny, M., Asa, C., Seal, U.S. and Mech, L.D. 1985. Chemical investigations of wolf (*Canis lupus*) anal-sac secretion in relation to breeding season. *Journal of Chemical Ecology*, 11, 593-608.

Remonti, L., Balestrieri, A., Smiroldo, G. and Prigioni, C. 2011. Scent marking of key food sources in the Eurasian otter. *Annales Zoologici Fennici*, 48, 287-294.

Röck, F., Barsan, N. and Weimar, U. 2008. Electronic Nose: Current status and future trends. *Chemical Reviews*, 108, 705-725.

Roberts, S.A., Simpson, D.M., Armstrong, S.D., Davidson, A.J., Robertson, D.H., McLean, L., Beynon, R.J. and Hurst, J.L. 2010. Darcin: A male pheromone that stimulates female memory and sexual attraction to an individual male's odour. *BMC Biology*, 8, 75.

Roos, A., Greyerz, E. Olsson, M. and Sandegren, F. 2001. The otter (*Lutra lutra*) in Sweden - population trends in relation to ΣDDT and total PCB concentrations during 1968-99. *Environmental Pollution*, 111, 457-469.

Rostain, R.R., Ben-David, M., Groves, P. and Randall, J.A. 2004. Why do river otters scent-mark? An experimental test of several hypotheses. *Animal Behaviour*, 68, 703-711.

Rosell, F., Jojola, S.M., Ingdal, K., Lassen, B.A., Swenson, J.E., Arnemo, J.M. and Zedrosser, A. 2011. Brown bears possess anal sacs and secretions may code for sex. *Journal of Zoology*, 283 (2), 143-152.

Rosell, F. and Steifetten, O. 2004. Subspecies discrimination in the Scandinavian beaver (*Castor fiber*): combining behavioral and chemical evidence. *Canadian Journal of Zoology*, 82 (6), 902-909.

Rosell, F. and Sundsdal, L.J. 2001. Ordorant source used in Eurasian beaver territory marking. *Journal of Chemical Ecology*, 27(12), 2471-2491.

Rozhnov, V.V. and Rogoschik, B. 1994. The ability of the river otter (*Lutra lutra L.)* to distinguish fresh scent marks and longevity of conserved scent mark information. *Lutreola*, 3, 5-9.

Rozhnov, V.V. and Rozhnov, Y.V. 2003. Roles of different types of excretions in mediated communication by scent marks of the common palm civet, *Paradoxurus hermaphroditus* Pallas, 1777 (Mammalia, Carnivora). *Biology Bulletin*, 30(6), 584-590.

Safi K. and Kerth G. 2003. Secretions of the interaural gland contain information about individuality and colony membership in the Bechstein's bat. *Animal Behaviour*, 65, 363-369.

Sanz, C., Ansorena, D., Bello, J. and Cid, C.C. 2001. Optimizing headspace temperature and time sampling for identification of volatile compounds in ground roasted Arabica coffee. *Journal of Agricultural and Food Chemistry*, 49, 1364-1369.

Schaefer, M.L., Wongravee, K., Holmboe, M.E., Heinrich, N.M., Dixon, S.J., Zeskind, J.E., Kulaga, H.M., Brereton, R.G., Reed, R.R. and Trevejo, J.M. 2010. Mouse urinary biomarkers provide signatures of maturation, diet, stress level, and diurnal rhythm. *Chemical Senses*. 35: 459-471.

Schulte, B.A., Müller-Schwarze, D. and Sun, L. 1995. Using anal gland secretion to determine sex in beavers. *Journal of Wildlife Management,* 59, 614–618.

Shumake, S.A. 1977. The search for applications of chemical signals in wildlife management. In: Müller-Schwarze, D. and Mozell, M.M eds. *Chemical Signals in Vertebrates*. New York: Plenum Press, pp. 357-376.

Schwartz, M.K., Luikart, G. and Waples, R.S. 2007. Genetic monitoring as a promising tool for conservation and management. *Trends in Ecology and Evolution,* 22, 25-33.

Schwarzenberger, F. 2007. The many uses of non-invasive faecal steroid monitoring in zoo and wildlife species. *International Zoo Yearbook*, 41, 52-74.

Schwende F.J., Wiesler D., Jorgenson J.W., Carmack M. and Novotny M. 1986. Urinary volatile constituents of the house mouse, *Mus musculus*, and their endocrine dependency. *Journal of Chemical Ecology*, 12, 277-296.

Scordato, E.S., Dubay, G. and Drea, C.M. 2007. Chemical composition of scent marks in the ringtailed lemur (*Lemur catta*): Glandular differences, seasonal variation, and individual signatures. *Chemical Senses*, 32, 493-504.

Service, K.M., Brereton, R.G. and Harris, S. 2001. Analysis of badger urine volatiles using gas chromatography-mass spectrometry and pattern recognition techniques. *Analyst*, 126, 615-623.

Setchell, J.M., Vaglio, S., Moggi-Cecchi, J., Boscaro, F., Calamai, L. and Knapp, L.A. 2010. Chemical composition of scent-gland secretions in an old world monkey (*Mandrillus sphinx*): Influence of sex, male status, and individual identity. *Chemical Senses*, 35, 205-220.

Shaikh AA, Shaikh, S.A., Celaya, C.L. and Gomez, I. 1982. Temporal relationship of hormonal peaks to ovulation and sex skin deturgescence in the baboon. *Primates*, 23(3):444-452.

Sherrard-Smith, E. and Chadwick, E.A. 2010. Age structure of the otter (*Lutra lutra*) population in England and Wales, and problems with cementum ageing. *IUCN Otter Specialist Group Bulletin*, 27, 42-49.

Simpson, V.R. and Coxon, K.E. 2000. Intraspecific aggression, cannibalism and suspected infanticide in otters. *British Wildlife*, 11, 423-426.

Singer, A.G., Beauchamp, G.K. and Yamazaki, K. 1997. Volatile signals of the major histocompatibility complex in male mouse urine. *Proceedings of the National Academy of Sciences of the United States of America*, 94, 2210-2214.

Sinkowski, G. 2001. *Scent communication in Mustelids.* Honours dissertation, Cardiff University.

Smith, T.E., Tomlinson, A.J., Mlotkiewicz, J.A. and Abbott, D.H. 2001. Female marmoset monkeys (*Callithrix jacchus*) can be identified from the chemical composition of their scent marks. *Chemical Senses*, 26, 449-458.

Strachan, R. 2007. *National survey of otter* Lutra lutra *distribution in Scotland 2003–04.* Scottish Natural Heritage Commissioned Report No. 211 (ROAME No. F03AC309).

Strachan, R. 2010. *Otter survey of Wales 2009-2010 Technical Summary*. Cardiff: Environment Agency Wales.

Statheropoulosa, M., Spiliopouloub, C. and Agapioua, A. 2005. A study of volatile organic compounds evolved from the decaying human body. *Forensic Science International*, 153, 147–155.

Stevens, R. 2000. *Anal scent gland secretion of the otter* Lutra lutra. Honours dissertation, Cardiff University.

Stockley, P. and Bro-Jørgensen, J. 2011. Female competition and its evolutionary consequences in mammals. *Biological Reviews*, 86, 341–366.

Stoddart, D. M., Aplin, R. T. and Wood, M. J. 1975. Evidence for social difference in the flank organ secretion of *Arvicola terrestris* (Rodentia: Microtinae). *Journal of Zoology*, 177 (4), 529-540.

Spurr, E.B., Ragg, J.R., O'Connor, C.E., Hamilton, W.J., Moller, H., Woolhouse, A.D., Morse, C.W., Morriss, G.A., Arnold, G.C. and Clapperton, B.K. 2004. Effect of concentration of anal gland scent lures on the capture rate of ferrets (*Mustela furo*) in winter and spring. *New Zealand Journal of Zoology*, 31(3), 227-232.

Sulkava, R. 2007. Snow tracking: a relevant method for estimating otter *Lutra lutra* populations. *Wildlife Biology*, 13, 208-218.

Sun, L. and Müller-Schwarze, D. 1998a. Anal gland secretion codes for family membership in the beaver. *Behavioral Ecology and Sociobiology*, 44, 199-208.

Sun, L. and Müller-Schwarze, D. 1998b. Anal gland secretion codes for relatedness in the beaver, *Castor canadensis*. *Ethology*, 104, 917-927.

Swaisgood, R.R., Lindburg, D.G. and Zhou, X.P. 1999. Giant pandas discriminate individual differences in conspecific scent. *Animal Behaviour*, 57, 1045-1053.

Swaisgood, R.R., Lindburg, D.G. and Zhang H. 2002. Discrimination of oestrous status in giant pandas (*Ailuropoda melanoleuca*) via chemical cues in urine. *Journal of Zoology*, 257, 381-386.

Taberlet, P. and Luikart, G. 1999. Non invasive genetic sampling and individual identification. *Biological Journal of the Linnean Society*, 68, 41-55.

Thom M.D. and Hurst, J.L. 2004. Individual Recognition by scent. *Annales Zoologici Fennici*, 41, 765-787.

Tibbetts, E.A. and Dale, J. 2007. Individual recognition: it is good to be different. *Trends in Ecology and Evolution*, 22, 529-537.

Trowbridge B.J. 1983. *Olfactory communication in the European otter (*Lutra lutra*)*. PhD Thesis, University of Aberdeen.

Tschirch, W., Hempel, G., Rothman, H., Schipke, R. and Klenke, R. 1996. Fäkalsteroiduntersuchungen. In: Artenschutzprogramm Fischotter in Sachsen. Landesamt fü Umwelt und geologie, p32-34.

Ward, A.J.W., Duff, A.J., Horsfall, J.S. and Currie, S. 2008. Scents and scents-ability: Pollution disrupts chemical social recognition and shoaling in fish. *Proceedings of the Royal Society B: Biological Sciences*, 275, 101-105.

Wasser, S., Risler, L. and Steiner, R. 1988. Excreted steroids in primate feces over the menstrual cycle and pregnancy. *Biology of Reproduction*, 55, 393-399.

Wasser, S.K. Davenport, B., Ramage, E.R., Hunt,K.E., Parker, M., Clarke, C. and Stenhouse, G. 2004. Scat detection dogs in wildlife research and management: application to grizzly and black bears in the Yellowhead Ecosystem, Alberta, Canada. *Canadian Journal of Zoology*, 82, 475–492.

White, A.M., Swaisgood,R.R. and Zhang, H. 2003. Chemical communication in the giant panda (*Ailuropoda melanoleuca*): the role of age in the signaller and assessor. *Journal of Zoology*, 259, 171-178.

Mark J. Whittingham, M, J, Stephens, P.A., Bradbury, R.B. and Freckleton , R.P. 2006. Why Do We Still Use Stepwise Modelling in Ecology and Behaviour? *Journal of Animal Ecology*, 75, 1182-1189.

Willse, A., Belcher, A.M., Preti, G., Wahl, J.H., Thresher, M., Yang, P., Yamazaki, K. and Beauchamp, G.K. 2005. Identification of major histocompatibility complex-regulated body odorants by statistical analysis of a comparative gas chromatography/mass spectrometry experiment. *Analytical Chemistry*, 77, 2348-2361.

Wilson, G.J. and Delahay, R.J. 2001. A review of methods to estimate the abundance of terrestrial carnivores using field signs and observation. *Wildlife Research*, 28, 151-164.

Wood, W.F., Terwilliger, M.N. and Copeland, J.P. 2005. Volatile compounds from anal glands of the wolverine, *Gulo gulo*. *Journal of Chemical Ecology*, 31, 2111-2117.

Woodley, S.K. and Baum, M.J. 2003. Effects of sex hormones and gender on attraction thresholds for volatile anal scent gland odors in ferrets. *Hormones and Behavior*, 44, 110-118.

Wyatt, T.D. 2003. *Pheremones and animal behaviour*. Cambridge: Cambridge University Press.

Yoxon, P. 1998. Spraint surveys and sparsely populated otter populations. *IUCN Otter Specialist Group Bulletin*. 15(2), 109-111.

Yuan, H., Liu, D., Sun, L., Wei, R., Zhang, G. and Su, R. 2004. Anogenital gland secretions code for sex and age in the giant panda, *Ailuropoda melanoleuca*. *Canadian Journal of Zoology*, 82, 1596-1604.

Zhang, JX., Zhang, ZB. and Wang, ZW. 2001. Scent, social status, and reproductive condition in rat-like hamsters (*Cricetulus triton*). *Physiology & Benavior*, 74 (4-5), 415- 420.

Zhang, J.X., Sun, L., Zhang, Z-B., Wang, Z-W., Chen, Y. and Wang, R. 2002a. Volatile compounds in anal gland of Siberian Weasels (*Mustela sibirica*) and Steppe polecats (*M. eversmanni*). *Journal of Chemical Ecology*, 28, 1287-1297.

Zhang, J-X., Zhang, Z-B., Wang, R., Chen, Y., Cao X-P., Wang F-S. and Wang Z-W. 2002b. A headspace sampling method and its application in analysis of volatiles of anal glands of the American mink (*Mustela vision*). *Acta Zoologica Sinica*, 48, 415-419.

Zhang, J.X., Ni, J., Ren, X.J., Sun, L., Zhang, Z.B. and Wang, Z.W. 2003. Possible coding for recognition of sexes, individuals and species in anal gland volatiles of *Mustela eversmanni* and *M. sibirica*. *Chemical Senses*, 28, 318-388.

Zhang J.X., Soini H.A., Bruce K.E., Wiesler D., Woodley S.K., Baum M.J. and Novotny M.V. 2005. Putative chemosignals of the ferret (*Mustela furo*) associated with individual and gender recognition. *Chemical Senses*, 30, 727-737.

Zhu, H.O., Lu, Z.G., Cai, J.H., Li, J.J. and Gao, L.R. 2009. Development of a headspace-SPME-GC/MS method to determine volatile organic compounds released from textiles. *Polymer Testing*, 28, 521-527.

Zomer, S., Dixon, S.J., Xu, Y., Jensen, S.P., Wang, H., Lanyon, C.V., O'Donnell, A.G., Clare, A.S., Gosling, L.M., Penn, D.J. and Brereton R.G. 2009. Consensus multivariate methods in gas chromatography mass spectrometry and denaturing gradient gel electrophoresis: MHC-congenic and other strains of mice can be classified according to the profiles of volatiles and microflora in their scent-marks. *Analyst*, 134, 114-123.

Appendix 1. Provisionally identified volatile organic compounds found in otter anal gland secretions, faeces and spraint.

Table 1. VOCs provisionally identified in otter anal glands (n=158) in Chapter 3 and 4. 164 further unidentified compounds were detected and used in analyses.

Aliphatic hydrocarbons
2-Methyl-2-butene
3,5,5-Trimethyl-2-hexene
2-Methylheptane
5-Ethyl-2-methylheptane
Octane
(E)-2-Octene
(Z)-2-Octene
1,3-Octadiene
2-Methyloctane
1,3-Trans,5-cis-octatriene
3,7-Dimethyl-1-octene
2,6-Dimethyloctane
Nonane
5-(2-Methylpropyl)nonane
2-Methylnonane
cis-4-Decene
Decane
4-Methyldecane
4-Methylenedecane
2-Methyldecane
Undecane
2,9-Undecadiene
Dodecane
7,11-Dimethyl-3-methylene-(Z)-1,6,10-dodecatriene
Tridecane
Tetradecane
Pentadecane
2,6,10,14-Tetramethyl-pentadecane
Hexadecane

Alicyclic hydrocarbons
1-Propylcyclopropane
(1-Methoxy-pentyl)cyclopropane
3-Propylcyclopentene
Butylcyclopentane
1,2,4-Trimethylcyclohexane
E,Z-4-Ethylidenecyclohexene
1-Ethyl-4-methylcyclohexane
Propylcyclohexane
4-Propyl-cyclohexene
7-Propylidene-bicyclo[4.1.0]heptane

Aromatic hydrocarbons
Toluene
Ethylbenzene
p-Xylene
Styrene
2-Ethenylnaphthalene

Alcohols, phenols and ethers
2-Methyl-1-butanol
1-Pentanol
2-Penten-1-ol
3-Methyl-2-buten-1-ol
2,4-Dimethylcyclopentanol
4-Methyl-1-pentanol
1-Hexanol
2,6-Dimethyl-4-heptanol
3-Methyl-1-hexanol
1-Octen-3-ol
3-Octanol
2,4-Dimethylcyclohexanol
Benzyl Alcohol
trans-(2-Ethylcyclopentyl)methanol
2-Octen-1-ol
1-Octanol
Cyclooctanemethanol
1-Adamantanol
Phenylethyl alcohol
1-Methoxyadamantane
1-(3-Hydroxymethyl-phenyl)-heptan-1-ol
Phenol
Methylphenol
Dimethylphenol
Ethylphenol
Ethylmethylphenol
Pentylphenol

Acids
2-Methylpropanoic acid
Butanoic acid
3-Methylbutanoic acid
2-Methylbutanoic acid
Pentanoic acid
Hexanoic acid anhydride

Aldehydes

2-Pentenal
3-Methyl-2-butenal
Hexanal
2-Ethyl-2-butenal
Heptanal
Benzaldehyde
Octanal
2,4-Heptadienal
5-Ethylcyclopent-1-enecarboxaldehyde
2-Hydroxybenzaldehyde
Benzeneacetaldehyde
2-Octenal
3,7-Dimethyl-2,6-octadienal
2-Isopropyl-4-methylhex-2-enal
2-Isopropyl-5-methylhex-2-enal
Nonanal
3-Ethylbenzaldehyde
2-Nonenal
2,6,6-Trimethyl-1-cyclohexene-1-carboxaldehyde
2,4-Decadienal
2-Butyl-2-octenal
5-Methyl-2-phenyl-2-hexenal

Oxygenous heterocyclic compounds

2-Propylfuran
2-Butylfuran
2-Pentylfuran
3-Pentylfuran
2-(1-Pentenyl)furan
2-(2-Pentenyl)furan
2-Hexylfuran
2-Acetyl-5-methylfuran
5-Ethenyltetrahydro-5-trimethyl-2-furanmethanol
Dihydro-2-methyl-3(2H)furanone
5-Acetyldihydro-2(3H)furanone
5-Ethenyldihydro-5-methyl-2(3H)furanone
Dihydro-5-pentyl-2(3H)furanone
5-Methyl-2-(2-methyl-2-tetrahydrofuryl)tetrahydrofuran
1-(2-Furanyl)ethanone
5,6,7,7-Tetrahydro-4,4,7-trimethyl-2(4H)benzofuranone
Pentyloxirane
(2S,6R,7S,8E)-(+)-2,7-Epoxy-4,8-megastigmadiene
Linanool oxide
Methyl-[4-methyl-3-pentenyl]oxiranemethanol
5,6-Dihydro-4-(2-methyl-1-propen-3-yl)-2H-pyran-2-one
3,7-Bis[(trimethylsilyl)oxy]-9-methoxy-1-methyl(6H)dibenzo[b,d]pyran-6-one

Nitrogenous compounds

2,6-Bis(2-chlorophenyl)-benzo[1,2-c:4,5-c']dipyrrole-1,3,5,7(2H,6H)tetrone
Isopropylimidazole-2-thione
1,2-Dicarboxy-3-(4-chlorophenyl)-2,3(1H)-dihydropyrido(1,2-a)benzimidazole
4,5-Dihydro-3-methyl-1,2,4-oxadiazine-5-one
1-Isopropyl-5-methyl-2-pyrazoline
2,3-Dimethylpyrazine
3-Methylpyridazine
Indole
3-[(2,3,4,9-Tetrahydro-1H-pyrido[3,4-b]indol-1-yl)methyl]-2-cyclohexen-1-one
2-(4-Iodo-phenyl)-6-pentyl-5,6,7,8-tetrahydro-quinoline
7-Chloro-2,3-dihydro-3-(4-N,N-dimethylaminobenzylidene)-5-phenyl-1H-1,4-benzodiazepin-2-one
1,1-Diethylhydrazine
1,1-Diethyl-2-(1-methylethyl)hydrazine
Acetic acid N'-[3-(1-hydroxy-1-phenylethyl)phenyl] hydrazide
Methoxy-phenyl-oxime
N-(Phenylmethylene)methanamine
N-(Phenylmethylene)-1-propanamine
3-Methyl-N-(3-methylbutylidene)-1-butanamine
3-Methyl-N-(2-phenylethylidene)-1-butanamine
N-(Phenylmethylene)-benzeneethanamine
Benzonitrile
2-Methyl-2-nitropropane

Sulphurous compounds

1-(Methylthio)-2-methylbut-2-ene
1-(Methylthio)pentane
1-(Methylthio)hexane
(Methylthio)cyclohexane
1-(Methylthio)heptane
3-(Methylthio)2-butanone
1-(Methylthio)3-pentanone
2-Ethylthiophene
2-Pentylthiophene
S-Methyl 2-methylpropanethioate
S-Methyl 3-methylbutanethioate
S-Methyl hexanethioate
Trans-hexahydro-cyclopent[e]-1,3-oxazine-2(3H)-thione
N-Morpholinomethyl-isopropyl-sulfide
Dimethyldisulfide
Methylethyldisulfide
Isopentylmethyldisulfide
2,4-Dithiapentane
Methyl (methylthio)methyldisulfide
Dimethyltrisulfide

Ketones

3-Hexanone
3-Hydroxy-2-butanone
3-Methyl-2-pentanone
2,4-Pentanedione
2-Hexanone
4-(Acetyloxy)-3-penten-2-one
4-Methyl-2-pentanone
5-Methyl-3-hexanone
Cyclohexanone
3-Hexen-2-one
1-(1-Cyclohexen-1-yl)ethanone
2-Heptanone
1-Chloro-3,3-dimethyl-2-butanone
2-Ethylcyclopentanone
5-Methylhexane-2,4-dione
2,3-Heptanedione
4-Methyl-3-methylene-1-oxetan-2-one
3-Ethylcyclopentanone
3-Octanone
6-Methyl-5-hepten-2-one
2-Octanone
4-Ethylcyclohexanone
2,2,6-Trimethylcyclohexanone
3-Octen-2-one
Acetophenone
3,5-Octadien-2-one
5,6-Decanedione
3-Nonanone
2-Nonanone
3,5,5-Trimethyl-2-cyclopenten-1-one
Benzylmethylketone
1-Phenyl-1,2-propanedione
2-(2-Nitro-2-propenyl)cyclohexanone
4-Methyl-3-heptanone
4-Acetyl-2,3,4,5,5-pentamethyl-2-cyclopenten-1-one
2-Pentylcyclopentanone
4-Butylcyclohexanone
2,2,6,6-Tetramethyl-3,5-heptanedione
1-Phenyl-2-hexanone
6,10-Dimethyl-5,9-undecadien-2-one
7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione

Esters

Acetic acid 2-methylpropyl ester
Acetic acid 3-methyl-1-butyl ester
Propanoic acid propyl ester
Propanoic acid 1-methylethyl ester
Propanoic acid butyl ester
Propanoic acid 2-methylpropyl ester
Propanoic acid 2-methylbutyl ester
Propanoic acid 3-methylbutyl ester
2-Methyl-propanoic acid ethyl ester
2-Methyl-propanoic acid propyl ester
2-Methyl-propanoic acid 2-methylpropyl ester
2-Methyl-propanoic acid 3-methylbutyl ester
2-Methyl-propanoic acid 2-methylbutyl ester
2-Methyl-propanoic acid 2,2-dimethyl-1-(2-hydroxy-1-methylethyl)propyl ester
2-Methyl-propanoic acid hexyl ester
Butanoic acid ethyl ester
Butanoic acid propyl ester
Butanoic acid 1-methylethyl ester
Butanoic acid butyl ester
Butanoic acid 1-methylpropyl ester
Butanoic acid 2-methylpropyl ester
Butanoic acid 1,1-dimethylethyl ester
Butanoic acid 2-pentenyl ester
Butanoic acid phenylethyl ester
2-Methyl-butanoic acid ethyl ester
2-Methyl-butanoic acid propyl ester
2-Methyl-butanoic acid 1-methylethyl ester
2-Methyl-butanoic acid 2-methylpropyl ester
2-Methyl-butanoic acid pentyl ester
2-Methyl-butanoic acid 2-methylbutyl ester
2-Methyl-butanoic acid hexyl ester
3-Methyl-butanoic acid methyl ester
3-Methyl-butanoic acid ethyl ester
3-Methyl-butanoic acid propyl ester
3-Methyl-butanoic acid 1-methylethyl ester
3-Methyl-butanoic acid butyl ester
3-Methyl-butanoic acid 2-methylpropyl ester
3-Methyl-butanoic acid pentyl ester
3-Methyl-butanoic acid 2-methylbutyl ester
3-Methyl-butanoic acid 3-methylbutyl ester
3-Methyl-butanoic acid hexyl ester
Pentanoic acid methyl ester
Pentanoic acid ethyl ester
4-Methyl-pentanoic acid methyl ester
4-Methyl-pentanoic acid ethyl ester
5-Hydroxy-pentanoic acid 2,4-di-t-butylphenyl ester
Hexanoic acid methyl ester
Hexanoic acid ethyl ester
5-Methyl-hexanoic acid ethyl ester
Hexanoic acid vinyl ester
Hexanoic acid isopentyl ester
Heptanoic acid 3-buten-1-yl ester
Octanoic acid ethyl ester
Dodecanedioic acid bis(tert-butyldimethylsilyl) ester
N-(3-furanylcarbonyl)-glycine methyl ester
15-Hydroxy-7-oxodehydroabietic acid methyl ester 15-trimethylsilyl ether
Benzeneacetic acid methyl ester
Benzeneacetic acid ethyl ester
Benzeneacetic acid 4-chlorophenyl ester
Benzenepropanoic acid ethyl ester
Benzenepropanoic acid isopropyl ester
Benzenepropanoic acid 3-methylbutyl ester
2-Trifluoromethylbenzoic acid 2-chlorophenyl ester
1,3-Benzenediol monobenzoate

Table 2. VOCs provisionally identified in otter faeces (n=10) in Chapter 3 that were not present in anal gland samples.

Name	Occurrence
1-(2-Aminophenyl)pyrrole	1
1-Azabicyclo[3.1.0]hexane	1
2-Methyl-N-(2-methylbutylidene)-1-butanamine	1
3-Methyl-1-butanamine	1
3-Methyl-1-butanol	5
2,3-Dihydro-1,2-dimethyl-1H-indole	1
1-Penten-3-ol	1
2-(Diethylamino)-1-phenyl-1-propanone	1
2,4,7,9-Tetramethyl-5-decyn-4,7-diol	1
2-Acetoxy-3-methoxybiphenylene	1
3-Methyl-2-butanone	2
2-Methyl-2-butenal	2
2-Ethyl-2-hexenal	1
2-Methyl-1-butene	2
2-Oxo-5-benzoyl-4,6-diphenyl-1,2,3,4-tetrahydropyrimidine	1
2-Pentanone	1
1-Butoxy-2-propanol	2
3,4-Dihydroxy-5-methoxybenzaldehyde	2
2-Methyl-3-hexanone	1
3-Pentanone	1
5-Isopropyl-2,4-imidazolidinedione	1
6-[2-Hexahydroazepino-1-hydroxyethyl]-8-methyl-2-p-methylphenylquinoline	1
Acetic anhydride	1
1-Vinyl-aziridine	1
N,N-diethyl-benzenamine	1
1,3-Dichloro-4-(4-methoxybenzylidenamino)-benzene	1
α-Ethylidene-benzeneacetaldehyde,	1
2,6-Bis(2-chlorophenyl)-benzo[1,2-c:4,5-c']dipyrrole-1,3,5,7(2H,6H)-tetrone	1
2-Methyl-butanenitrile	1
3-Methyl-butanenitrile	1
Dimethyldiazene	1
2,2'-(octahydro-2,3-quinoxalinediylidene)bis[1-phenyl]-ethanone	1
Ethyl 4-(ethyloxy)-2-oxobut-3-enoate	1
Ethyl isocyanide	1
2-Ethyl- furan	1
Heptadecane	1
Hexadecanal	1
3,3-Dimethyl-hexane	1
Hexanethioic acid S-methyl ester	1
n-Caproic acid vinyl ester	2
Nonadecane	1
Oxalic acid diallyl ester	1
Cis-2-(1,1-dimethylethyl)-3-ethyl-oxirane,	1
Pentadecanal	1
Pentanal	1
3-Methyl-pentane	1
3-(methylthio)-propanal	1
2-methyl-propanoic acid 1-(1,1-dimethylethyl)-2-methyl-1,3-propanediyl ester	3
2,6-Dimethyl- pyrazine,	1
Trimethylpyrazine	1
6-(4-chlorophenyl)-5-phenylazo-pyridazine-3,4(3H,4H)-dione	1

Pyridine	2
2,3,4,5-Tetrahydro-pyridine	3
Pyrrole	5
3,9-Dimethylundecane	1
4,7-Dimethylundecane	1

Table 3. VOCs provisionally identified in otter spraint (n=47) in Chapter 5.
72 further compounds not identified were detected and used in analyses.

Retention index	Compound	Occurrence	Mean relative abundance	SD
743	(S) 2-Methyl-1-butanol	35	1.16	1.33
748	Acetic acid	3	5.43	6.98
754	3-Undecanone	11	1.17	1.36
755	3-Ethyl-2,2-dimethyl-oxirane	6	0.23	0.18
757	n-Propyl acetate	7	0.92	1.26
768	2-Methyl-2-butenal	4	0.49	0.39
769	Dimethyldisulfide	35	3.84	9.28
772	1-Pentanol	43	6.78	9.89
773	Pyridine	3	0.16	0.19
774	(E)2-Pentenal	8	1.29	1.26
775	3-Methyl-2-pentanone	5	0.10	0.04
776	Pyrrole	21	0.56	0.70
777	3,3'-Oxybiscyclopentene	7	0.35	0.27
780	2,2,3-Trimethylcyclobutanone	18	0.55	0.48
784	(Z) 2-Penten-1-ol	26	0.52	0.73
786	1-Hexen-3-ol	4	0.45	0.33
794	2,3-Hexanedione	18	0.28	0.32
795	Methyl isobutyl ketone	6	0.03	0.01
798	trans-(2-Chlorovinyl)dimethylethoxysilane	36	0.23	0.20
800	Hexanal	42	2.69	5.46
802	Butanoic acid ethyl ester	10	1.73	3.60
819	Butanoic acid	14	0.61	0.81
830	Acetyl valeryl	7	0.08	0.06
833	2-methyl-2-pentenal	5	2.80	3.26
847	3-Methyl-butanoic acid ethyl ester	6	1.40	2.13
856	Ethylbenzene	34	0.32	0.24
861	3-Methyl- butanoic acid	6	0.22	0.19
863	4-Methyl-1-pentanol	33	0.43	0.35
875	3-Methyl-1-butanol acetate	5	0.14	0.15
876	Hexanenitrile	4	0.08	0.06
884	Styrene	39	1.46	1.44
890	2-Heptanone	35	0.29	0.19
900	4-Heptenal	20	2.55	5.38
902	Heptanal	26	0.75	1.02
922	Methoxy-phenyl-oxime-,	45	6.56	5.83
928	Butanoic acid propyl ester	6	2.14	3.07
939	1-Butoxy-2-propanol,	10	0.41	0.66
949	(E)-1-(2-butenyloxy)-pentane	5	0.05	0.02
949	2-Ethyl- hexanal	3	0.10	0.06
951	6-Methyl-2-heptanone	5	0.10	0.07
952	Benzaldehyde	43	21.79	24.24
956	Dimethyl trisulfide	19	3.54	8.71
966	Acetic acid, N'-[3-(1-hydroxy-1-phenylethyl)phenyl]hydrazide	46	0.64	0.64

971	3,5,5-Trimethyl-2-hexene	37	1.68	2.49
973	Aniline	13	12.49	15.11
978	Benzonitrile	3	0.05	0.02
978	1-Octen-3-ol	42	0.89	0.75
984	8-Heptadecene	5	0.06	0.04
986	3-Octanone	7	0.48	0.26
986	1,3,5-Trimethyl-benzene	3	0.09	0.06
986	6-Methyl-5-hepten-2-one	30	0.49	0.55
989	2-Pentyl-furan	11	0.10	0.06
990	Phenol	10	5.66	8.48
992	2-Octanone	7	0.09	0.06
1002	4-Ethyl-cyclohexanone	3	0.05	0.05
1003	Octanal	6	0.07	0.04
1015	2,2,4,6,6-Pentamethyl- heptane	15	4.51	4.33
1026	2,2,6-Trimethyl-cyclohexanone	21	0.18	0.13
1031	2-Ethyl-1-hexanol	42	9.76	14.06
1047	2-Methyl-propanoic acid 2-methylpropyl ester	3	0.14	0.08
1055	(E)2-Octenal	4	0.29	0.31
1057	4,4-Dimethyl-cyclohex-2-en-1-ol	3	0.06	0.05
1069	(E)2-Octen-1-ol	13	0.29	0.26
1070	2,8-Dimethyl-undecane	4	0.03	0.03
1078	3,5-Octadien-2-one	7	2.49	5.06
1093	2-Nonanone	29	0.29	0.28
1104	Nonanal	9	0.59	0.57
1138	2,6,6-Trimethyl-2-cyclohexene-1,4-dione	6	0.13	0.08
1151	(E,E)2,6-Nonadienal	4	1.72	1.36
1185	3-Ethyl-3-methylheptane	15	0.14	0.15
1238	1-Iodoundecane	7	0.03	0.01
1286	Indole	41	13.64	13.45
1295	2-Undecanone	7	0.18	0.15
1345	2-methyl-propanoic acid 2,2-dimethyl-1-(2-hydroxy-1-methylethyl)propyl ester	14	0.41	0.29
1379	2-methyl-propanoic acid 3-hydroxy-2,4,4-trimethylpentyl ester	27	0.42	0.41
1461	6,10-Dimethyl-5,9-undecadien-2-one	21	0.19	0.15
1463	(E)6,10-Dimethyl-5,9-undecadien-2-one	10	0.03	0.04
1467	2,6-Bis(1,1-dimethylethyl)-2,5-cyclohexadiene-1,4-dione	20	3.73	6.89
1486	(Z)-6-Octen-2-one	6	0.94	0.70
1500	Pentadecane	44	0.86	1.45
1500	1-Tridecanol	12	0.17	0.30
1551	7-Methyl-pentadecane	3	0.04	0.03
1573	3-Methyl-pentadecane	4	0.04	0.02
1579	Dodecanedioic acid bis(tert-butyldimethylsilyl) ester	44	0.23	0.20
1598	2-methyl- propanoic acid 1-(1,1-dimethylethyl)-2-methyl-1,3-propanediyl ester	16	0.55	0.71
1694	2,6-Bis(1,1-dimethylethyl)-4-(1-methylpropyl)-phenol	3	0.08	0.01
1707	2,6,10,14-Tetramethyl- pentadecane	30	1.38	1.85
1745	7-Methyl- heptadecane	7	0.19	0.19
1777	3,5-di-tert-Butyl-4-hydroxybenzaldehyde	10	0.19	0.25
1871	1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethyl-cyclopenta[g]-2-benzopyran	28	0.09	0.07