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Chemical Communication in the European Otter, *Lutra lutra*

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by

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CONTENTS

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ABSTRACT

European otter (*Lutra lutra*) scent marks (*spraint* and *anal sac secretion* material) from captive and wild animals were analysed using solvent extraction and solid phase micro extraction (SPME). An ageing study (using SPME) mapped spraint chemical composition changes following deposition and how low temperature storage and exposure to the environment affected these changes. Chemicals with opposite time dependence were found; providing a possible spraint ageing mechanism. Temperature and environmental conditions affected the rates of production and deterioration of these chemicals and therefore the accuracy of spraint age estimations. Identification of the fatty acid content of otter scent marking material from 4 otter species led to both intra-specific (*L.lutra*) and inter-specific (Canadian river, *Lontra canadensis*; Asian short claw, *Aonyx cinerea*; and Giant, *Pteronura brasiliensis*) comparisons. Low volatility fatty acids (C_{10}-$C_{24.1}$) were found in all scent mark types. Intra-specifically, differentiation was seen in spraint fatty acid profiles based on sexual identity. Differentiation was observed between *L. lutra* scent marking material types (Captive spraint; Wild spraint; Wild anal sac secretion). Inter-specific differences relied on a mixture of 'digital' and 'analogue' coding. SPME and gas chromatography – mass spectrometry (GCMS) were used to investigate intra- (*L.lutra*) and inter- specific (*L. lutra*, *A. cinerea*, *L. canadensis*, and badger, *Meles meles*) differences in the headspace chemicals of otter scent marks. Inter-sanctuary (possibly diet related) differences in spraint odour of *L. lutra* were seen. No *overall* male - female differences were found, although possible intra-sanctuary sexual differences were seen. Inter-specific differentiation in scent mark odour profiles due to a combination of both analogue and digital coding elements was observed.

CHAPTER 1

LITERATURE REVIEW

SCENT MARKING IN MUSTELIDS

Transmission of information between individuals is essential for the maintenance of the complex social lives of carnivores including that of the mustelid family. The means of transferring such information can differ, the main routes being sound (e.g., vocalization) (Harrington & Mech, 1979; Newton-Fisher et al, 1993), vision (e.g., cues such as facial expression or body language) (Fox, 1970; Kruuk, 1976), touch (e.g., more direct contact through fighting and grooming) (Bekoff, 1974; White & Harris, 1994) and of course odour (Hutchings & White, 2000).

Olfactory signalling in carnivores often plays a key role in intra-specific communication (Beauchamp et al, 1976; Gorman, 1980; Kranz, 1996; Rostain et al, 2004). The use of odour signalling can be more useful than other methods especially under circumstances where visual and auditory signals are hard to detect, e.g., at night or underground (Gorman & Trowbridge, 1989). A record of an animal's spatial and temporal movements can be provided with the deposition of a scent mark (Kruuk, 1995), which is designed to persist in the environment long after the signalling animal has moved on (Bradbury & Vehrencamp, 1998).

The function of scent marking

What is the precise function of scent marking? There are a number of hypotheses concerned with this question.

Some focus on its role in the maintenance and defence of territories (Gosling, 1982; Gosling & McKay, 1990). They put forward the idea that scent marks convey information about a territory resident. Territoriality in mammals often appears where resources are limited, and so in energetic terms, it is important for individuals to minimize the energy expended in defending it, whilst still maintaining an effective defence (Hutchings & White, 2000).

Defence of a territory using scent dramatically reduces the chances of direct contact/conflict between rivals. The potential risks involved in physical confrontation are substantial (Maynard-Smith & Parker, 1976; Rubenstein, 1982) and so scent marking

brings with it many advantages (Hutchings & White, 2000). Individual recognition through scent marks has been shown to exist in some species of carnivore (Gorman, 1976; Gorman & Trowbridge, 1989) and not only could this provide the mechanism for a strategy of avoidance, but also could be utilised in mediation of encounters between members of the same group (Hutchings & White, 2000). Scent marks therefore provide a low risk means of intra- and inter-territorial communication.

Many mustelids concentrate their scent-marking deposition on or near to territorial boundaries (Hutchings & White, 2000). Badgers (*Meles meles*) living at high population density, for example, were shown to have latrines placed on paths around their territory borders (Kruuk, 1978a). This type of scent mark deposition pattern has also been documented in high-density badger populations in other UK areas (Brown, 1993; Roper et al, 1993) and low-density badger populations in Italy (Pigozzi, 1990).

Scent marking is also often observed at particular places inside mustelid territory, for instance on conspicuous objects (Kruuk, 1995), at the point where paths dissect a specific linear feature (White, Brown & Harris, 1993), or at the junctions of paths (Kruuk et al, 1984). The specific positioning of these hinterland scent marks implies that they could also function as *intra*-group communications. So, just as scent marking could prevent *inter*-group encounters between individuals, so too could it perform a similar role in the prevention of *intra*-group encounters (Hutchings & White, 2000). In UK badgers, scent marks have been observed concentrated around the main sett. Roper et al (1993) suggests these hinterland scent marks play a defensive role. Pine martens (*Martes martes*) have a similar scent marking pattern to that of the badger, i.e., concentrated at borders and in central parts of the territory (Pulliainen, 1982).

Sex specific differences in scent mark deposition have been investigated in badgers (Brown, 1993; Roper et al, 1993). Hinterland latrines were visited by male and female badgers with approximately the same frequency. Border latrines however, were visited much more regularly by male badgers especially during the breeding period and this may act partly as a deterrent to neighbouring rival males (Hutchings & White, 2000).

In the North American river otter, *Lontra canadensis*, males explored other male spraints more than those of anoestrous females (Rostain et al, 2004) indicating that scent marking

may possibly play a role in social communication (in coastal North American river otter populations males are more social than females) (Blundell et al, 2002a). Dominant males showed a greater interest in investigating the spraints of other males whether they were familiar or not (Rostain et al, 2004).

In the wild, dominant males of both mustelid and non-mustleid species tend to scent mark more and also to overmark subordinate scent (e.g., the stoat, *Mustela erminea,* Erlinge et al, 1982; the European badger*,* Kruuk et al, 1984; the aardwolf, *Proteles cristatus,* Silwa & Richardson, 1998). It would therefore follow that a dominant animal would be more interested than a subordinate in investigating the scent of other individuals (Rostain et al, 2004). Scent marking has also been implicated in the maintenance of dominance hierarchies in other mustelids. Dominant male badgers not only scent mark at a higher frequency than other clan members, but also squat mark other clan members more often and in particular oestrous females (Kruuk et al, 1984). Similarly, in the ferret (*Mustela furo*) dominant males scent mark more than subordinates and will overmark scent produced by subordinates (Clapperton, 1989). Both dominant male and female stoats mark more frequently than their subordinates (Erlinge et al, 1982). Upon encountering a dominant scent mark a subordinate stoat will use submissive vocalisations or avoid it altogether (Erlinge et al, 1982). Managing dominance relationships in this way is valuable and it has been shown in other types of carnivore to minimize the costs of aggressive encounters (the coyote, *Canis latrans,* Gese & Ruff, 1997; felidae, Macdonald et al, 1998; the wolf, *Canis lupus*, Mech, 1999).

Rostain et al (2004) also found that North American river otters spent equal amounts of time investigating both familiar and unfamiliar male scent. One explanation might be that, in coastal populations, there are seasonal and yearly variations in the composition of the group (Blundell et al, 2002 a, b). Therefore, Rostain et al (2004) were not able to conclude that scent marking in the North American river otters is territorial. This seems to agree with studies on the function of sprainting in the European otter, *Lutra lutra* (Durbin, 1989; Kruuk, 1995).

Group formation in coastal populations seems to be driven more by advantages gained from cooperative fishing than kinship (Blundell et al, 2002a). Rostain et al (2004) found

no influence from relatedness on results of familiar vs unfamiliar preference tests and they conclude that kin recognition from spraints may not hold any significance for group interactions.

Coastal wild-caught adult male otters underwent a series of preference tests and were found to spend more time investigating spraints from conspecifics than those from sealions. Similarly, in marsh living river otter populations, otters visit conspecific scent in preference to that of mink, *Mustela vison* (Humphrey & Zinn, 1982). Spraints are therefore also likely to signal species identity.

Sources of odour

Relatively little is known about the natural scent marking patterns of many mustelid species (Hutchings & White, 2000). There are many sources of odour utilized by mustelids. Odour may comprise chemicals provided by food, synthesized by the animal or produced via bacteria (often, scent organs are moist, warm and anaerobic – perfect conditions for bacterial development).

Urine and faeces

Urine and faeces have been demonstrated to be significant contributors to communication through scent (Service, 1997).

Urine

For many species of mustelid, urine is used as part of a scent marking strategy. Examples include the stoat, and the weasel, *Musela nivalis* (Erlinge et al, 1982; King, 1989); the mink, (MacLennan & Bailey, 1969; Brinck et al, 1978; Dunstone, 1993); the pine marten, *Martes martes* (Pulliainen, 1982; de Monte & Roeder, 1990); the otter, (Kruuk & Hewson, 1978; Kruuk, 1995); the badger, (Service et al, 2001); and the wolverine, *Gulo gulo* (Koehler et al, 1980).

At high densities, badgers mostly deposit their urine at latrines (Brown, 1993). At low densities, badgers will deposit urine in discrete patches and 96% of urinations are unconnected with latrines (Hutchings, 1996). The honey badger, *Mellivora capensis*

deposits urine in large amounts at latrines (eliminatory function), but also in token amounts in holes along the foraging path (communicative function – thought to maintain spatio-temporal separation) (Begg et al, 2003). Urine is deposited at latrines by the ferret *Mustela furo* (Clapperton, 1989), singly by mink, (Birks, 1981) and often deposited in conjunction with anal drag by the stoat (Erlinge et al, 1982). Chemical analysis of volatiles contained in badger urine has revealed the possibility that it conveys information on age, sex, reproductive status and individual identity (Service, 1997; Service et al, 2001).

Faeces

Faeces are also widely used by mustelids in their scent marking strategy. Examples include the stoat and the weasel (Erlinge et al, 1982; King, 1989); the mink (MacLennan & Bailey, 1969; Brinck et al, 1978; Dunstone, 1993); the European otter (Gorman et al, 1978; Kruuk & Hewson, 1978; Kruuk, 1995); the badger, (Roper et al, 1986; Roper et al, 1993); and the wolverine (Koehler et al, 1980).

At low population densities, badgers will deposit faeces singly, either in dung pits or directly onto the ground (Pigozzi, 1990; Brown, 1993). At high densities, badgers will deposit faeces at latrines (Kruuk, 1978a; Roper et al, 1986; Brown, 1993). Latrines are shared defecation sites, which are thought to have a remote olfactory communication function important in maintaining badger spacing system (Kruuk 1978a). At latrines, badgers sometimes also deposit secretions from their anal glands on top of the faeces (Roper et al, 1986). The ferret deposits faeces (together with urine and anal sac secretion) at latrines (Clapperton, 1989).

Faeces are often deposited singly by mustelid species and very often at elevated or prominent positions. The pine marten will deposit scats on stones or tufts of grass (Pulliainen, 1982) and the otter will also spraint at vantage points, such as prominent rocks along the water's edge, under bridges or near trees (Kruuk, 1995). Sprainting behaviour in otters is thought to signal use of vital resources (e.g., food, freshwater pools in coastal habitat) and to maintain spatio-temporal separation (Kruuk, 1995).

However, in the wolverine defecation appears to be inactive in terms of scent marking, although occasionally individuals urinate onto older scats, which then function as scent posts (Magoun, 1985). Polecat (*Mustela putorius*) scats are also not considered to be used in scent marking as they are normally deposited discreetly in den-associated latrines (Corbet & Harris, 1991).

Scent producing organs

There are a variety of scent organs seen in mustelids, from simple increases in the size and density of skin glands to complex anatomical structures. Scent glands are mostly found on the face, the tail and the perineum (especially the anal region).

The anal and tail region

Every European mustelid possesses anal sacs or glands (Pocock, 1921). Stubbe (1970) described 4 different types of mustelid anal sac according to genera (*Mustela, Martes Meles*, and *Lutra*). Those in *Mustela and Martes* were found to be more sophisticated with high concentrations of tubular and alveolar glands.

Mustela

Many species in the *Mustela* share similar anal sac anatomy and deposit their secretions in a similar manner. For example, in the mink and the stoat, anal sac secretion is expelled via ducts terminating just internal to the anus, but separated from the rectum (Brinck et al, 1978; Erlinge et al, 1982). The secretion is thought to be deposited on objects through a process called 'anal dragging' and also via body rubbing (mink, Brinck et al, 1978; stoat, Erlinge et al, 1982; and weasel, Erlinge, 1995). In the stoat, 'anal drag' involves an individual moving forward using mainly its front legs, whilst dragging the pelvis area along the substrate; its tail in a raised, curled position (Erlinge at al, 1982). In the weasel, it is achieved by depressing the anal region and slowly moving forwards or backwards. In this way anal sac secretion is deposited on the substrate.

The anal sac secretion is described in mink as being yellow and turbid, consisting of immiscible lipid and aqueous phases (Sokolov et al, 1980) and as a viscous, yellow secretion in the stoat (Crump, 1978).

Anal sac secretion is thought to be used in territorial marking within the *Mustela*. Stoat, anal sac secretion is deposited via anal drag onto stones, tree branches or bare ground and is accompanied by urination. Anal drag is used to demarcate the home range, mark new objects and over-mark scent from conspecifics and is performed throughout the year, regardless of the breeding season and by both sexes (Erlinge et al, 1982).

Mink use scats to mark their territories, but active object marking using anal drag has occasionally been observed in the field (Brinck et al, 1978). Mink also utilise their anal sac secretion during aggressive encounters, when the tail is uplifted and moved sideways, presumably to disperse the odour of the anal sac secretion (Brinck et al, 1978).

In New Zealand the ferret is nocturnal and solitary and both males and females maintain their separate, but overlapping territories using scent marking from a number of different sources (proctodael glands; preputial glands; epitricheal tubular glands; sebaceous skin glands and anal sacs) (Moors & Lavers, 1981; Clapperton, 1985, 1989).

Social communication in *Mustela* includes a contribution from anal sac secretion which has been shown to contain information allowing both sex and individual recognition in stoats and ferrets (Erlinge et al, 1982; Kelliher & Baum, 2002; Woodley and Baum, 2003, 2004). In a study by Clapperton et al (1988) indoles were shown to be more abundant in the anal sac secretion of the male ferret when compared with the female ferret. However, a subsequent study by Zhang, et al (2005) revealed no such difference in indole concentration between the sexes.

The genus *Mustela* is the most intensively studied group in the Mustelidae with reference to chemical communication. Odours are known to emit from the anal sacs and anal sac secretion has been chemically analysed in seven *Mustela* species (the mink (Brinck et al, 1978, 1983; Sokolov et al, 1980); the stoat (Crump, 1980b; Brinck et al, 1983); the domestic ferret, *Mustela putorius furo* (Crump, 1980a; Crump and Moors, 1985; Zhang et al, 2005); the European polecat (Brinck et al, 1983); the steppe polecat, *Mustela eversmanni* (Zhang et al, 2002, 2003); the Siberian weasel, *Mustela sibirica* (Zhang et al, 2002, 2003) and the weasel (Brinck et al, 1983; Buglass et al, 1991). The majority of volatile compounds identified in the anal sac secretion of these seven species were sulphur containing compounds unique to the genus (Brinck et al, 1983; Crump & Moors,

1985, Zhang et al, 2002, 2003) [see Table 1]. There were also 2 nitrogen-containing compounds (indole and *o*-amnioacetophenone). *O*-amnioacetophenone was found only in four of the species investigated (the stoat, Brinck et al, 1983; the domestic ferret, Zhang et al, 2005; the steppe polecat, and the Siberian weasel, Zhang et al 2003) whereas indole was found in every species investigated, although not by every study. This may be explained by the fact that differing analytical methods are possibly responsible for composition differences of volatile chemicals found in complex biological media (Zhang et al, 2005). The type of sample extraction/preparation procedure used can have a large effect on which compounds are identified (Soini et al, 2005). For example, the Zhang et al (2005) study into ferret chemosignals used a relatively new sampling technique for the determination of chemical profiles – headspace stir bar sorptive extraction followed by thermal desorption GC-MS. This resulted in 10 volatiles (two aldehydes, five ketones, benzothiazole, 2-methylquinoline and 4-methylquinazoline), previously unidentified, being discovered in ferret anal sac secretion.

Table 1: Comparison of studies into anal sac secretion chemical analysis in the genus *Mustela*.

		Mustela vison			***Mustela erminea***		***Mustela putorius furo***			***Mustela putorius***	***Mustela eversmanni admirati***		***Mustela sibirica fortanieri***		***Mustela nivalis***	
		Brinck et al, 1978	**Sokolov et al, 1980**	**Brinck et al, 1983**	**Crump 1980b**	**Brinck et al, 1983**	**Crump, 1980a**	**Crump & Moors, 1985**	**Zhang et al, 2005**	**Brinck et al, 1983**	**Zhang et al, 2002**	**Zhang et al, 2003**	**Zhang et al, 2002**	**Zhang et al, 2003**	**Brinck et al, 1983**	**Buglass et al, 1991**
Extraction method (all analysed using GC/MS)	**Solvent**	n-pentane		methylene chloride	diethyl ether	methylene chloride	diethyl ether			methylene chloride		dichloro methane		dichloro methane	methylene chloride	
	Headspace		RND[1]						HSSE[2]							
Sample number		5♂5♀	pooled same-sex samples	3♂	???	34♂16♀	???		3♂3♀	8♂7♀		11♂10♀		11♂11♀	23♂19♀	
Sample storage temperature (°C)		-30	-20	-20	-10	-20	-10		-20	-20		-20		-20	-20	
Compound																
acetophenone									✓							
***o*-amnioacetophenone**						✓			✓(♂)			✓		✓		
benzaldehyde									✓							
benzothiazole									✓							
dimethyl disulphide			✓													
2,2-dimethylthiacyclobutane		✓	✓													
1,2-dithiacycloheptane		✓														
3,3-dimethyl-1,2-dithiacyclopentane			✓													
2,2-dimethylthietane				✓		✓	✓		✓		✓	✓	✓	✓		
(Z)- or (E)- 2,4- dimethylthietane									✓		✓	✓	✓	✓		
3,3-dimethyl-1,2-dithiolane							✓		✓							
3,3-dimethyl-1,2-dithiacyclopentane				✓						✓	✓	✓	✓	✓	✓	
(E)-3,4-dimethyl-1,2-dithiacyclopentane												✓				
(Z)-3,4-dimethyl-1,2-dithiacyclopentane											✓	✓		✓		
(E)-3,4-dimethyl-1,2-dithiolane									✓							
(Z)-3,4-dimethyl-1,2-dithiolane									✓							
(E)-2,3-dimethylthietane									✓		✓	✓	✓	✓		
***cis*-2,3-dimethylthietane**							✓									
***trans*-2,3-dimethylthietane**						✓	✓			✓					✓	
***cis*- or *trans*- 2,4-dimethylthietane**															✓	
3,3-dimethyldithiolane																✓
***cis*-3,4-dimethyldithiolane**																✓

***trans*-3,4-dimethyldithiolane**															✓
2-ethylthietane			✓	✓(♀)	✓			✓1		✓	✓	✓	✓		
3-ethyl-1,2-dithiacyclopentane					✓						✓		✓		
3-ethyl-1,2-dithiolane				✓(♀)				✓(♀)							
(E)-2-ethyl-3-methylthietane								✓1		✓	✓		✓		
(Z)-2-ethyl-3-methylthietane								✓		✓			✓		
geranylacetone								✓							
indole	✓		✓	✓	✓	✓		✓	✓		✓		✓	✓	
isopentyl methyl sulphide		✓													
2-isopropyl thietane							✓	✓			✓		✓		
6-methyl-5-hepten-2-one								✓							
2-methylquinoline								✓							
4-methylquinazoline								✓							
2-octanone								✓							
nonanal								✓							
2-pentylthietane				✓	✓	✓		✓			✓				
2-propylthietane				✓	✓	✓		✓		✓	✓	✓	✓		
2-propylthietane, isomer of					✓				✓						
2-propylthietane, isomer of					✓										
3-propyl-1,2-dithiacyclopentane					✓										
3- or 4-propyl-1,2-dithiacyclopentane											✓				
4- or 3-propyl-1,2-dithiacyclopentane											✓		✓		
3-propyl-1,2-dithiolane				✓		✓		✓							
putrescine		✓													
quinoline						✓		✓							
S-methyl thiocetate		✓													
unknown isomer 2,2-dimethylthiacyclobutane	✓														
unknown NON-suphur-containing compound				✓(♂)											
unknown sulphur-containing compound				✓											

[1] – RND (Ranay Nickel Desulfuration) [2] – HSSE (Headspace Stirbar Extraction) ??? – sample numbers not specified

Martes

Pine martens deposit their anal sac secretion by rubbing against the target item or substrate (Corbet & Harris, 1991). Anal secretion is used to mark areas throughout the pine marten territory (Schwanz, 2000).

The sulphur-containing compounds typically found in the *Mustela* species do not occur in the anal sac secretion of the pine marten (Brinck et al, 1983; Schildknecht & Birkner, 1983). This is also true of the beech marten (Schildknecht & Birkner, 1983).

Benzaldehyde was identified in pine marten anal sac secretion (Brinck et al, 1983) and short-chain carboxylic acids were found in both pine marten and beech marten (Schildknecht & Birkner, 1983).

Meles

Badger anal sacs are paired structures found just internal to the anus (Stubbe, 1971). The sacs act as reservoirs for an odourous, orange-coloured secretion which is deposited at latrines in association with faeces (Neal, 1977; Roper, Shepherdson & Davies, 1986). Latrines and anal sac secretion are thought to function in territoriality (Neal, 1977; Kruuk, 1978a; Roper et al, 1986; Stewart et al, 2001).

Badger sub-caudal gland secretion has been described as 'highly volatile' by Gorman, Kruuk & Leitch (1984) and they suggest a short term signalling function (e.g., to signal behavioural intent). This seems at odds with the territorial role proposed by other authors for anal sac secretion.

Davies et al (1988) found that overall, the anal sac secretion scent profiles of males and females (whether wild or captive) appeared similar. This suggested that all badgers may have a 'species-specific' anal secretion scent profile. Indeed, to the human nose at least, all samples had the same musky odour typical of badgers. No consistent sexual or individual differences were found. There was a suggestion that anal secretion could be affected by environmental aspects such as living conditions or diet with the scent profiles of two badger populations being significantly different.

Unlike that of a number of different carnivores (Gorman, Nedwell & Smith, 1974; Albone & Perry; 1976; Preti et al, 1976; Sokolov et al, 1980) the anal sac secretion of the badger contained no volatile fatty acids (Davies et al, 1988). All those identified in the secretion are high molecular weight fatty acids of low volatility in the range C_{14}-C_{24}.

Due to the presence of these long chain fatty acids of low volatility, badger (*Meles meles*) anal gland secretion would be well suited to long term signalling and may function in badger territoriality (Davies et al, 1988).

The European badger is the only European mustelid known to possess a sub-caudal gland of such size (Hutchings & White, 2000). First described by Stubbe (1971), it consists of a substantial skin invagination between the root of the tail and the anus, forming a relatively hairless pouch; opening via a 20-80mm horizontal slit. Sebaceous (holocrine) and sudoriferous (apocrine) glands empty their lipid-rich, paste-like secretion into the pouch for storage until deposition (Gorman et al, 1984; Hutchings & White, 2000; Buesching et al, 2002a). Sub-caudal secretion is used to scent mark the rump and flanks of conspecifics (Gorman et al, 1984; Hutchings & White, 2000; Buesching et al, 2002a), the ground around their sett and paths (Gorman et al, 1984; Buesching et al, 2002a), bedding material plus other objects and latrines (Buesching et al, 2002a).

There have been many suggestions for the function of badger subcaudal secretion:

- territorial signalling (Kruuk, 1978a)
- group membership (Gorman et al, 1984)
- individual recognition (Kruuk et al, 1984)
- fitness advertisement (Buesching & Macdonald, 2001)

Buesching et al (2002a) investigated the chemical profile of the subcaudal secretions using GC-MS. In total, 110 compounds were found, with 21 of these common to all samples and 6 of these were positively identified (see Table 2)

Table 2. Positively identified compounds found in subcaudal secretion of the badger using solvent extraction and gc-ms (Buesching et al, 2002a)

Compounds found in subcaudal secretion of the badger
dodecanoic acid
tetradecanoic acid
hexadecanoic acid
heptadecanoic acid
linoleic acid
octodecanoic acid

The subcaudal secretion chemical profile is highly individual specific, however no single peak was found to be typical for any of the categories investigated (i.e., group membership, season,

sex, age, body condition) (Buesching et al, 2002a). The secretion is thought to code for group membership and individuality, and possibly also may convey information regarding fitness-related parameters (Buesching et al, 2002a).

Gulo

A tannish-yellow, highly odoriferous secretion is produced by the paired anal glands of the wolverine (Hash, 1987 in Wood et al, 2005).

Chemicals in the secretion were solvent extracted and analysed using GC-MS (Wood et al, 2005). Categories of compound included: *carboxylic acids; alcohols; hydrocarbons; nitrogen compounds* and *others*. Overall, 123 peaks were seen, but there was great variation between individuals with the number of peaks per animal ranging from 45 - 71.

59 compounds were identified, 10 tentatively and only 6 of these were common to all samples:

- 3-methylbutanoic acid
- 2-methylbutanoic acid
- phenylacetic acid
- 2-methyldecanoic acid (tentative identification only)
- α-tocopherol
- cholesterol.

5 compounds were common to all but one of the samples:

- hexadecanoic acid
- linoleic acid
- stearic acid
- 5,6,7,7-tetrahydro-2(4H) benzofuran (tentative identification only)
- 1,2-hexadecanediol (tentative identification only)

2 compounds were common to all but two of the samples:

- pentadecanoic
- (Z)-9-hexadecenoic acids were

Wolverine anal sac secretion has a strong odour despite the absence of the types of sulphur-containing compounds (thietanes and dithiolanes) found in the *Mustela* species. Short-chain carboxylic acids and phenylacetic acid found in all extracts contribute to this strong odour. Other ‘smelly’ constituents found in some, but not all, of the samples include dimethyl disulfide;

dimethyl trisulfide and 3-phenylpropanoic acid. Low and non-volatile compounds (including free fatty acids and cholesterol) were also present.

Therefore, the chemical profile of wolverine anal sac secretion is similar to that of the pine marten and beech marten. The sulphur-containing compounds typically found in the *Mustela* species do not occur in the anal sac secretion of the pine and beech marten (Brinck et al, 1983; Schildknecht & Birkner, 1983). Benzaldehyde was identified in pine marten anal sac secretion (Brinck et al, 1983) and short-chain carboxylic acids were found in both pine marten and beech marten (Schildknecht & Birkner, 1983).

Wolverines and martens are more closely related (phylogenetically) than either species is to the genus *Mustela* (Dragoo & Honeycutt, 1997; Koepfli & Wayne, 2003; Marmi et al, 2004)

Other types of gland

There is evidence to show that ventral glands are present in mustelids (Macdonald, 1985) and also that the composition of the secretion they produce is distinct from that of the anal glands (Brinck et al, 1983). It has been demonstrated stoats and weasels use such glands for marking objects. Behavioural trials have demonstrated a subordinate cowering response in stoats when confronted with anal or ventral gland deposition, implying that both are able to provide individual identity and dominance status information.

Other types of gland found in mustelids include chin glands - found in sables, *Martes zibelina* and inter-digital glands – badgers have inter-digital glands that are thought to assist in the scent marking of a territory, in particular along badger paths (Neal & Cheeseman, 1996). Scraping behaviour is seen in otters with vegetation being removed from areas of up to 1m in width; this is associated with sprainting. Is this simply a visual signal supplementing the olfactory scent mark or does the otter also possess inter-digital scent glands?

THE EUROPEAN OTTER

Introduction

Otters belong to the mustelid sub-family Lutrinae which contains at least thirteen species of otter, including the species investigated in this study, the European or Eurasian otter *Lutra lutra* (Linnaeus, 1758). See Figure 1.

Figure 1. The European or Eurasiasn otter (Photograph, taken by Mr. Richard Dodd at Tamar Otter Sanctuary, North Petherwin, Cornwall, UK).

Otters are well adapted to their semi aquatic lifestyle, with their long, sinuous bodies, webbed feet and muscular tail (Woodroffe, 1994). Thick fur together with a relatively high metabolic rate helps to maintain their body temperature when submerged (Chanin, 1993). These adaptations however represent a compromise; otters must be sufficiently accomplished in water to be able to catch fish in their natural environment (otters are mainly piscivorous), whilst being reasonably proficient at moving around on land, where they spend a large proportion of their time (Chanin, 1993).

When moving around on land, an otter relies largely on olfactory and visual stimuli. Its vision on land is poor in comparison to its ability underwater, which, in bright conditions is better than most animals (Balliet & Shusterman, 1971; Schusterman & Barret, 1973). At night or where underwater visibility is impaired, otters rely more on their vibrissae (whiskers) to detect and to pursue prey (Green, 1977).

As the otter is solitary and largely nocturnal, visual and vocal signals are poorly developed (Trowbridge, 1983). If otters do ever meet, there is very little visual display and almost no

change in facial expression (Kruuk, 1995); this has also been seen in other otter species (Duplaix-Hall, 1975; Duplaix, 1980) and also in the badger (Kruuk, 1995). This may be a general trait of mustelids that contrasts with, e.g., facial expressions shown by canids (Fox, 1970; Light et al, 1993).

Sound communication plays a minor role in the otter. Most sounds produced by this species fail to travel very long distances, with the exception of the contact call between mother and offspring – a bird-like whistle – that carries over several hundred metres (Kruuk, 1995).

Travelling over short distances on land, their sense of smell provides otters with much more information; they constantly sniff the air as they move. During an encounter between individuals on land the otter approaching from upwind will invariably move to a downwind position of the other individual in order to pick up its scent (Kruuk, 1995).

Otter status

As its name suggests, The European or Eurasian otter has a huge geographical distribution (larger than that of any other otter species). Its range stretches from the United Kingdom and Europe in the west, to the rain forests of Asia in the east and from northern Africa, India and Indonesia up into northern Lapland and Siberia (Woodroffe, 1994).

There are, however, obvious gaps in the otter's former range, where it has declined (especially since the 1950s). For instance, in Britain otters are commonly found in Scotland, Wales and Ireland, but are now absent from much of southern and central England. Indeed, the species is now rare or absent from most of central Europe (Macdonald 1995).

Otters are secretive animals and occur at low densities with a nocturnal pattern being typical of those living on rivers (contrasting with coastline populations that are active during the day). This meant that otter decline was largely missed (Mason & Macdonald, 1986). Ironically, concern over the level of the Eurasian otter population in Britain was initially expressed by hunters of the animal (Chanin 1993) and the decline was confirmed by a subsequent report commissioned by the Mammal Society entitled '*The Otter in Britain*' (Anon, 1969). Data from otter hunts was used and demonstrated a significant drop in hunting success between 1957 and 1967. With this discovery, hunting gradually diminished and in 1978 all hunting ceased when the otter was added to the schedule of endangered species and protected by law (they were also included in the Wildlife and Countryside Act of 1981).

Strategies other than hunt records were needed for assessment and monitoring of population numbers and the publicity surrounding the otter's decline prompted a series of population surveys. Funding from the Nature Conservancy Council, Royal Society for Nature Conservation (RSNC) and the Vincent Wildlife Trust supported national surveys (*England*: [*]Lenton et al, 1980; *Wales*: [†]Crawford et al, 1979– see Table 3; *Scotland*: Green & Green, 1980; *Ireland*: Chapman & Chapman, 1982). The findings of these surveys reinforced concern over the level of the otter population in Britain.

These initial surveys in England and Wales were followed by 3 more (see Table 3) and the 2002 survey for both England and Wales confirmed the continued recovery and increase in otter distribution since the initial surveys were carried out.

Table 3. Percentage of positive sites for all full survey sites in 1977/8, 1984/5, 1991 and 2002.

	%age positive sites			
England	*1977/9*[*]	*1984/6*[**]	*1991-94*[***]	*2002*[****]
	5.8(5.8)	**8.9**(9.6)	**22.2**(23.4)	**34**(36.3)
Wales	*1977/8*[†]	*1984/5*[††]	*1991*[†††]	*2002*[††††]
	20(20)	**38**(38)	**53**(53)	**71**(74)

NB figures for sites common to all surveys are in brackets. Results expressed as percentage of survey sites with otter signs within alternate 50 km squares of the National Grid. For example in the 2000-02 survey, otter signs were found at 34% of the 3,327 sites visited.

[**] Strachan et al, 1990 / [***] Strachan & Jefferies, 1996 / [****] Crawford 2003
[††] Andrews & Crawford, 1986 / [†††] Andrews et al, 1993 / [††††] Jones & Jones 2004

This group of repeated surveys makes up the best historical data set for any UK mammal species.

This has been reflected elsewhere and there has been encouraging evidence that otter populations have also been increasing at different rates in countries bordering the Atlantic coast and parts of northern and eastern Europe, eastern Austria and regions of Norway (Green & Green, 1997).

Difficulties in surveying otter populations

The naturally low density of otter populations together with their elusive and shy nature makes direct observation of behaviour very difficult. For this reason field signs, particularly *spraints* (otter scent marks) have been used for many years in an attempt to help monitor otter populations, by establishing trends in both habitat preference and numbers (Green & Green, 1980/87/97; Jefferies, 1989; Jones & Jones, 2004).

To date this has been the best available monitoring method, but there has been some controversy, however over its validity. It has been argued that whilst spraints may be useful for monitoring otter distribution, their worth as a tool for monitoring otter numbers is limited. Kruuk et al (1986) advised caution in using spraint surveys as a method when assessing habitat utilisation by otters. Part of the rationale used for this argument was that spraint surveys are based on an untested assumption, i.e., that otters have spent more time in areas where there are more spraints. It was suggested that this might not be necessarily true, e.g., Kruuk had observed that otters sometimes spraint directly into watercourses (unpublished) and so the absence of spraints in an area may not necessarily imply the absence of otters. In a study of streams and lakes in the north east of Scotland, otter movements were radiotracked (Kruuk et al, 1998). Areas were identified in which otters spent a large proportion of time (e.g., reed marshes, far away from open water and along numerous small streams), but where no spraints were found. It has often been assumed that otters do not use bogs or small steams, due to the lack of spraints in these areas.

Chanin, 1993 suggests that this might be a strategy by females with young cubs. By not sprainting on land the females reduce the risk of predation on their cubs by infantacidal males. Infantacide is common to many carnivore species (Packer & Pusey, 1984).

Prigioni et al (1995) carried out an investigation of the sprainting habits of captive otters. The number of otters was found to have a relationship with scent marking levels. Prigioni et al (1995) contend that the scent marking level is useful when defining wild otter population status. However, it must be remembered that researchers collecting spraints from otters in a closed environment would be more likely recover the majority of the scent marks deposited. In a wild situation this would be much less likely. Indeed, Jenkins & Burrows (1980) state that only a limited proportion of the scent mark output of the wild otters in their study would have been recorded, as they probably only visited a section (and possibly only a limited section) of the total range. They conclude that the numbers of spraints found did not always provide a direct indication of the numbers of otters present. This demonstrates the possible inaccuracies of

extrapolating data provided by animals in captivity to wild animals in their natural environment and the difficulties of surveying wild populations.

With the validity of the available surveying methods being called into question, reliable information regarding otter populations is scarce. When the results from such surveys are used as the basis for conservation measures the need for a solid surveying technique is both evident and essential.

An alternative method available to estimate otter population size is injection of a harmless radionuclide (Zinc-65 isotope) which can be detected in deposited spraints using a scintillation counter (Kruuk et al, 1980; Kruuk, 1995). Individuals need to be trapped, injected with the isotope and then released. The proportion of labelled spraints in the area can then be assessed. However, this technique is also not without its problems:

- capture of wild otters has proved difficult (Mason & Macdonald, 1986)
- assumes labelled and non-labelled spraints have equal chances of discovery
- assumes that all individuals deposit spraints in equal amounts

A certain amount of success has been achieved utilising faecal and hair DNA fingerprinting, which has allowed the identification of some individual otters from spraints (Hansen & Jacobsen, 1999; Kohn & Wayne, 1997; Jansman et al, 2001; Huang et al, 2005). However, this work is time consuming, logistically difficult and extremely expensive in terms of manpower. DNA is only present in spraints in low amounts and is rapidly degraded when exposed to the elements and bacterial action. This leads to other problems including the need to use extremely fresh spraint material, collected early in the morning to prevent the degrading effects of increasing temperature (Hájková et al, 2006). Despite these problems spraint DNA typing shows promise as a tool for monitoring otter populations.

Spraints, anal glands and scent marking behaviour

Otter 'spraint' is a word used to describe scent marks deposited by the animal. The term was first used by hunters and is thought to originate from the French word *épreindre* – 'to squeeze out' (Chanin, 1993). Otters have two vesicular anal sacs lying either side of the rectum (Gorman et al, 1978), the ducts of which open into the alimentary canal allowing their contents to be smeared onto passing faeces (Bradshaw et al, 2001). Both the sacs and the ducts are lined with hairless, cornified epithelium, and beneath this, striated muscle is arranged around the duct in a dense dermis. Glandular products secreted into the duct can be squeezed up into the anal sacs by fine control of this muscle (Gorman et al, 1978). Sebaceous and sudoriferous glands lie in the connective tissue between each duct and its muscles and these glands secrete into the ducts and into the base of the anal sac itself. Secretion from these glands is stored in the lumen of the anal sacs along with cells sloughed from the epidermis (Gorman et al, 1978). The majority of the secretion inside the anal sac comprises protein and mucopolysaccharide from the apocrine glands, punctuated by lipid droplets from the sebaceous glands. Pigment granules in the apocrine secretion are responsible for its typically green, brown or yellow colour (Gorman et al, 1978). Stevens (2000, unpublished) describes anal sac secretions as dark brown, light brown, dark brown with white bits, light brown with white bits, or entirely white. Wild otter scent deposits are histochemically similar to anal sac secretion, but are 'contaminated' with faeces, urine and sometimes sperm (Gorman et al, 1978).

A fresh, wild otter spraint can vary from a small tar-like smear containing few or no bones, to cylindrical droppings 1cm in diameter and up to 10 cm in length, principally made up of fish bones and scales, crustacea shell, fur or feathers (Corbet & Harris, 1991). Spraints are usually dark and mucilaginous, but colour can vary from black or dark-greenish brown to very pale grey (Chanin 1993) and possess a unique odour, a musk-like scent that is their most effective diagnostic characteristic (Woodroffe, 1994). This distinctive smelling dollop of faeces, unequivocal proof of their presence, is the closest most people will ever get to an otter in its natural habitat (Kruuk, 1995).

The European otter scent marks throughout its home range or territory and spraints are deposited at prominent features of the landscape, (on top of conspicuous rocks near the water, under bridges, at tributary junctions) or at places of interest (small freshwater pools, near to holts). Great efforts are made by otters to visit such places on a regular basis (Kruuk, 1995). They may also scrape up soil, sand, gravel or vegetation to from a small heap, normally called a 'sign heap' upon which they deposit their spraint (Chanin, 1993). Scraping *without* heap formation

can also occur with vegetation removed from areas of up to 1m in width; this behaviour is also associated with sprainting (Corbet & Harris, 1991).

It has been suggested that spraints have a function other than that of elimination of undigested food, i.e., scent communication (Erlinge, 1968; Kruuk & Hewson, 1978; Mason & Macdonald, 1986; Kruuk, 1992). Sprainting sites, sometimes termed 'spraint piles' (Trowbridge, 1983; Gorman & Trowbridge, 1989) are often traditionally used for deposition and the high concentration of nitrogen encourages growth of nitrophilous plants (green algae along the coast and grasses like Yorkshire fog (*Holcus lanatus*) inland). The dark green colour of the plant growth adds to the already conspicuous nature of these sites.

Unlike otters, most other mustelids use skin gland secretions to mark themselves, conspecifics and objects directly. The otters' semi aquatic lifestyle may explain this difference as any secretions on the skin or pelt would be constantly washed away (Trowbridge, 1983).

Otter faeces ('spraints') are small and they are placed at particular, prominent sites on a regular basis (Erlinge, 1967b; Harris, 1968). Otters spend much time investigating the odour of both their own spraints and those of conspecifics. After an initial sniff, an individual will often turn through 180º and deposit its own spraint at the same place, overmarking a conspecifics odour or replenishing its own.

Trowbridge (1983) suggests the information most likely to be conveyed by otter scent marking would be:

- individual identity
- age
- sex
- sexual receptivity
- social status
- time since deposition

Trowbridge also contends that with this type of information an otter could potentially:

- demarcate its home range
- demarcate its territory
- avoid or meet other individuals

Simultaneous deposition of spraint and urine is sometimes observed, but the urine is produced in large volumes. So, it appears that spraint is the predominant medium responsible for olfactory communication, with urine retaining its eliminatory function (Trowbridge, 1983).

But, what is the purpose of otter scent marking? What is its biological function? Kruuk (1995) sought to answer some fundamental questions about sprainting behaviour, the most general of which being 'Why do otters spraint?' Four possible explanations follow:

Seasonal spraint deposition and sexual behaviour

Male and female differences would be expected if sprainting behaviour were connected in any way to sexual behaviour and would possibly be seasonal. Sprainting, therefore, might be expected to be most intense during the breeding season (Erlinge, 1968). Work carried out in Shetland revealed that spraint numbers varied greatly between visits, as did sprainting site utilisation along different stretches of coastline (Conroy & French, 1987). Significant *seasonal* fluctuation in spraint numbers was also seen with a ten-fold decrease in the number of spraints found per visit in summer compared to winter (Conroy & French, 1987). In Shetland otter populations, reproductive behaviour is seasonal (Kruuk et al, 1987; Kruuk 1995), so it would be reasonable to suggest a possible link between the two.

Similar seasonal patterns of spraint deposition have been seen in freshwater habitat along river systems in Wales (Macdonald & Mason, 1987) and Ireland (Ottino & Giller, 2000). In freshwater habitat however, reproduction can take place at any point in the year (Erlinge, 1967b; Harris, 1968; Kranz, 1996). Despite this, in Sweden, for example, otters tend to mate in late winter or early spring. Sprainting activity during this period was high, but not exceptionally high compared with other periods (Erlinge, 1968). This may place suggestions of a link between sprainting and reproduction in doubt.

An alternative explanation put forward is that otters spraint more often *on land* during the winter period (Kruuk, 1992): a definite 'seasonality' was found, particularly as winter gave way to spring. March saw a sprainting frequency 12 times higher than that seen in June. According to Kruuk, this was not due to fewer otters or less sprainting during the summer, but to an increase in sprainting directly into the water. All otters observed, from males to solitary females to females with cubs exhibited this behaviour.

Chanin (1985) suggests other possible reasons for this seasonal trend in spraint numbers; (i) the emergence of cubs into the environment, or (ii) reinforcement of otter dominance relationships within populations when newly independent young animals seek to establish home ranges of

their own. To support this, evidence of the presence of cubs in winter and spring was found in a number of studies on British river systems (in Wales – Macdonald & Mason, 1987; in Ireland - Ottino & Giller, 2000; in Scotland – Green et al, 1984). The suggestion is that young cubs still dependent on their mother start to patrol the territory under her supervision, resulting in higher territorial spraint deposition.

Seasonality of spraint numbers might also be explained by changing environmental conditions where the biggest threat to the longevity of the spraint is water, either from high humidity or rain. It may be that spraints simply get 'washed out' more during the winter, creating an increased requirement for scent mark replenishment.

Captive males have been found to scent mark with higher frequency than females (Hillegaart et al, 1985; Prigioni et al, 1995). In general scent marking levels seemed to be related to breeding activity. The lowest number of signs was observed for a month after the birth of a cub and the highest number of signs were recorded when the cubs were 5-6 months old. Prigioni et al (1995) state that this data closely agreed with the findings of Jenkins & Burrows (1980) who, in a study of wild otters on the river Dee in Aberdeenshire, attributed the presence of family groups to peaks in scent marking. Polotti et al (1995) reported that 5 month old cubs in captivity began to spraint following their mother and at 6 months independent activity was observed. Around this time the father has been observed to heavily increase its marking frequency (Prigioni et al, 1995). In the wild, young otters have been seen to spraint directly into the water (H.Watson – pers. comm. - reported in Jenkins & Burrows, 1980). Watson suggested that otters may spraint more on land after they are around 6 months old.

It is clear that, whilst extremely interesting, the situation that captive otters find themselves in is far from normal and this may influence the behaviour of these animals compared to their wild counterparts.

Otters often urinate at spraint sites and this could also function in communication between the sexes. It is known that cats and dogs can distinguish sex from urine (Verberne & de Boer 1976; Dunbar, 1977) and that female urine transmits information concerning oestrus in dogs (Doty & Dunbar, 1974). This was briefly investigated by Trowbridge (1983) and although chemical analysis showed a change in hormone concentration over time, there was no real evidence of a link with the oestrous cycle. Despite this, Trowbridge recommended further investigation, maintaining that urine remains the principle candidate for the signalling of female otter reproductive condition.

Territoriality

Otters live in solitary or group territories which have both aquatic and terrestrial elements (Kruuk et al, 1998). Otters may also occupy both marine and freshwater habitats within their home range:

Coastal otters

Work by Kruuk (1995) on Shetland otters showed more than one female can live within a particular area in so-called 'group ranges'. Within these ranges females seemed to have a preferred a 'core area' where they were observed most often. In general terms females occupying the same home range avoided each other spending most time in their core areas, however there was also a large degree of mutual tolerance elsewhere in the range. In contrast, females from neighbouring group ranges were aggressively excluded. Some core areas shifted from one year to the next, but the home range remained identical (Kruuk, 2006).

Male otters in these coastal regions are up to 1.5 times heavier than females and as in many other species (Clutton-Brock & Harvey, 1978) would be expected to have larger home ranges. The ranges of individual males in Shetland were larger and overlapped two or more female ranges (Erlinge, 1968; Kruuk, 1995) with several males using them simultaneously. The pattern of this overlap was not apparent, but males were much more aggressive towards one another than the females. As in other mustelid species (Powell, 1979) an aggressive, intrasexual territorial system seemed to define male otter dispersion.

Transient otters moved over greater distances than resident animals sometimes covering up to 40 km of coast. They also utilised inferior types of holts and food resources.

Freshwater otters

Otters were studied in freshwater habitats in 2 river systems (the valleys of the Dee and the Don) west of Aberdeen, Scotland (Kruuk et al, 1993; Durbin, 1993). Adult male otters were observed mostly on the main watercourses, occasionally moving up tributaries and around lakes, whilst the opposite was true of females, who spent most time along tributaries or in lakes. Encounters between radio-tracked and non radio-tracked otters were observed throughout their home ranges (not just at the boundaries). There was evidence that adult male home ranges encompassed more than one female range. In rivers and tributaries, female otters were found to be more solitary than marine otters, but shared territories on lakes; several females were seen to non-aggressively

use the same lake waters simultaneously, even when foraging with litters of cubs (Kruuk, 1995; Jenkins, 1980).

If sprainting functioned in territoriality then concentration of spraints at range borders might be expected (Gorman & Mills, 1984; Gorman & Trowbridge, 1989). Kruuk (1992) investigated a possible link between sprainting behaviour and territoriality in the otter population of Shetland. He looked at sprainting rates along four known territorial borders (an average of 0.26 spraints per landing) and compared them with sprainting rates elsewhere (0.37 spraints per landing) and found the differences not to be significantly different. This pattern of scent marking contrasts markedly with other carnivorous mammals, e.g., European badgers and spotted hyenas, where boundary scent marking is extensive (Kruuk, 1972, 1978a, 1989; Mills, 1990). The otter is more specialised than many other mustelid species and is restricted to particular types of habitat, which has an effect on territorial organisation. Many otters live alongside rivers and so their ranges are linear in shape. This is also true of those found living next to the coastline, e.g., in the West of Scotland and on Shetland (UK), where otters only used a relatively narrow strip of water and land along the coast (Kruuk, 1995). This means that an otter's territory is less defensible economically than, for instance, that of a badger and this is reflected in otter scent marking strategy. As an alternative to marking all the boundaries of their territories, otters limit their scent marks to particular areas within them.

Gosling (1982) puts forward his 'match hypothesis' regarding scent-marking behaviour. He argues that scent marks assist in territorial defence, particularly in a potentially aggressive encounter between a resident and a trespassing animal. Matching an individual's odour with that of the majority of the scent marks within the territory, the intruder is able to identify it as the resident. It is then in a position to assess the resident's investment in the territory, and will usually withdraw from any potential conflict. This prevents escalation of the encounter into a full-scale fight.

Kruuk (1995) contends that Gosling's hypothesis would probably not apply to otters. He qualifies this by explaining that, unlike many other carnivores (e.g., badgers and foxes) otters appear not to aggressively defend their territories, and that face-to-face interactions are rare. When male otters *do* meet there is almost invariably an aggressive physical encounter, but these incidences are rare (Kruuk, 2006). In autopsies on road kill otters from southern and south-west England, many animals (including mature males) have been shown to have injuries from violent encounters with other otters (Simpson, 2006).

Resource marking

Kruuk (1992) suggests an alternative explanation for the role of sprainting. It may act to signals to other otters that a particular stretch of coastline or river, is being, or has already been, utilised. Otters have been shown to have the ability to recognise individuals from their spraint odour (Gorman & Trowbridge, 1989), but this would not be necessary in order for this resource marking strategy to work (Kruuk, 1995).

Otters are creatures of habit and will utilise the same feeding sites, holts or freshwater washing pools over and over again. Advertising the fact that resources are being used in a particular area brings with it many advantages. If an otter arrives at a suitable feeding site and finds that it is in use, or has recently been used by another individual, it will benefit by moving on to another location that may be free of competition. This will also benefit the otter already using an area; it too will be able to forage without competition and can return to it in the knowledge that other individuals will not have fed there in its absence. The same holds true for other important otter resources, e.g., freshwater pools in coastal habitat. If too many individuals used it for washing then it would inevitably become salty and dirty so an otter indicates ownership of the pool by depositing scent marks nearby.

Kruuk (1995) has observed that in coastal otter populations:

- sprainting is carried out at approximately the same rate independent of sex or age
- sprainting is closely linked with fishing and eating
- sprainting is linked with holts and freshwater pools
- more sprainting occurs when resources are low. In both coastal (Kruuk et al, 1988) and freshwater (Kruuk et al, 1993, Erlinge, 1967a, 1968; Jenkins & Burrows, 1980, Mason & Macdonald, 1987) habitats there is high food availability in summer and a low amount of spraints and low food availability in winter / spring and a high amount of spraints.
- otters exploit ‘food patches’ rather than feeding indiscriminately throughout the territory. These become depleted, but stocks are replenished with potential prey every 24 hours. Otters have to consume large quantities of food compared with other carnivores due to the energetic costs of being in the water, swimming and diving (Kruuk, 1995). Kruuk’s observations suggest that otters in fresh water are living extremely close to their limits of possible existence. Therefore, there would be a definite benefit to a resident otter in marking such a food patch.

So, Kruuk's hypothesis is that sprainting advertises the use of a resource and serves as a dispersion mechanism that brings mutual benefit to all. In other words, otters may have developed a non-confrontational system, which allows partitioning of resource utilisation within their territory (Kruuk, 1995).

Otters in the Guadalete River, Spain, were also found to use the same strategy to signal the use of food resources. Available fish biomass was positively correlated with sprainting frequency (Prenda & Granado-Lorencio, 1996).

A study by Prigioni et al (2005) suggested that sprainting activity was linked to the distribution of fish availability. A positive correlation was found between the number of suitable sites for fish, such as pools or stretches of streams with good vegetation cover and sprainting intensity (spraints/km).

Durbin (1989) found that otters overmarked spraints whether or not they were from familiar individuals. A similar conclusion to that of Kruuk (1992) was reached, i.e., that otters may not be territorial in the classic sense with sprainting behaviour seeming to simply advertise an individual's presence in order to provide personal foraging space.

Individual identity

If scent material is used for individual recognition, then it needs to be stable when placed in the environment for periods of time that at least match the frequency that a scent mark will be encountered. If a scent mark is to be representative of an individual then it must possess this sort of stability (Albone & Perry, 1976; Albone et al, 1977; Preti et al, 1977).

There are three ways that individual (or indeed, sexual) identity might be conveyed:

1. *Digital* coding - unique individual scent compounds in each of an individual's spraints. For example, possibly similar to the sex specific scent components in the anal gland secretion of the African mongoose, *Herpestes ichneumon*. (Hefetz et al, 1984).
2. *Analogue* coding - spraints of every individual could comprise the same scent components with their relative proportions providing an individual odour 'fingerprint'.
3. A combination of both *Digital* and *Analogue* coding.

Sun & Müller-Schwarze (1998, 1999) first used the terms *digital* coding (presence or absence of compounds) and *analogue* coding (relative abundance of shared compounds) in their studies of anal sac secretion secretion in the North American beaver, *Castor canadensis*.

Work undertaken to show whether otters actually have the ability to discriminate between spraints from different individuals has produced conflicting results.

- A method of training associated with rewards was used to study the ability of 2 captive otters (1 male / 1 female) to discriminate between pairs of spraints in 2 separate categories (Trowbridge, 1980, 1983):
    - *individual's own spraints* vs *spraints from other individuals*
    - *familiar spraints* vs *unfamiliar spraints*

  The trained male otter was able to distinguish between spraints in both these categories.
- Durbin (1989) investigated the ability of wild otters to distinguish familiar and unfamiliar spraints using the same 2 categories used in the study by Trowbridge (1980, 1983); overmarking was used as an indirect measure of response. In contrast to the Trowbridge study no discrimination between spraints within the two categories was seen.
- Rozhnov and Rogoschik (1994) showed that captive otters (5 males / 4 females) were able to distinguish between their own spraints and those of other otter individuals. This confirms the result produced by Trowbridge (1983).

It might be assumed from these results that wild otters are able to discriminate between the odours of different spraints, but that the oversprainting response is not an indication of this. Durbin's study showed that strange and familiar spraints were both overmarked by the resident otter indicating that perhaps the purpose of sprainting was not territorial. Indeed, if sprainting was used in order to advertise the use of resources (Kruuk, 1992) and maintain spacing between individuals regardless of their familiarity then this indiscriminate overmarking behaviour would be expected. Questions must also be asked about the reliability of studies using low numbers of otters and also about the relevance of training otters to exhibit responses in exchange for rewards. Otters may have the ability to distinguish between spraints, but is this ability required or used in the wild situation.

If an individuality code within the spraint odour profiles could be deciphered revealing an individual odour 'fingerprint' and the age of a deposited spraint ascertained then this would prove an invaluable tool in monitoring both established otter populations and re-introduced or translocated animals, providing a far more accurate picture than simple spraint surveying.

Spraint ageing and time since deposition

The transmission of deposited scent marks is explored by Bradbury & Vehrencamp (1998). Deposited scent marks are designed to last a long time in the absence of the sender and emission rate and longevity of the olfactory signal can be substantially influenced by the following factors:

- *The chemical profile of the scent mark* – often, scent marks consist of sebaceous gland secretions and have active components embedded in a lipid-rich matrix that acts as a carrier. Increases in the polarity of the matrix result in a decrease in the speed of release of the active chemicals. For example, carboxylic acids are more likely to form hydrogen bonds with the matrix than non-polar hydrocarbons and so evaporate more slowly. The matrix also acts to a buffer the active components against substrate effects.
- *The type of substrate* – the following chemical and physical characteristics of the substrate can all affect the emission rate and longevity of scent marks (Regnier & Goodwin, 1977):
    - *surface area* – this determines the availability of the substrate binding sites for the active compounds.
    - *porosity* – this determines the rate of chemical release.
    - *chemical polarity* – the majority of organic scent mark compounds are polar and therefore adhere to natural surfaces that are made up of polar materials; this slows the emission rate.
- *The environmental conditions* – the following environmental factors can all affect the emission rate and longevity of scent marks
    - *wind and high temperature* – increased spread and fade-out of active compounds is seen by increasing diffusion and evaporation rates
    - *sun exposure* - scent mark chemical decomposition occurs with direct sun exposure. The scent mark matrix offers protection against both ultraviolet radiation and wind.
    - *high humidity and rain* - most detrimental environmental conditions in terms of longevity. Both will result in highly polar water molecules competing with the active odour molecules for the hydrogen binding sites in the carrier matrix. This is possibly a chemical signalling strategy to maximise longevity and release odour only in the presence of the receiving animal. Licking or breathing on the scent mark would introduce the water molecules necessary to provide the receiver with a concentrated 'hit' of active odour compounds. The unavoidable consequence of this

strategy is rapid fade-out of scent marks under these environmental conditions (Alberts 1992).

To date very few studies have investigated the effects of ageing on European otter spraints. A study of otters in Sweden gave an indication that otters might be able to discriminate between fresh and old spraints. However, this was based on just a single incident where a resident male was shot and its territory taken over by another male just 7 days later (Erlinge, 1967b, 1968).

Fasano & Milone (1993) carried out a chemical study into changes in the spraint odour profile in the period following deposition. Thin Layer Chomatography was used to study the chemical composition (12/24/48 hours after deposition). Esters were shown to disappear whilst sterols and fatty acids remained constant providing a possible mechanism for distinguishing the age of a spraint.

Rozhnov & Rogoschik (1994) investigated the longevity of conserved scent marks in otters. Having established the test otters' ability to distinguish between their own fresh spraints and other fresh spraints, this was used as the criteria to assess the spraints effectiveness over time (15/30/60 days after deposition). This study was unable to show whether or not otters were able to distinguish between fresh and old spraints as the otters had a similar reaction to both. However, it was shown that otters can discriminate between their own old spraints and other old spraints even 60 days following deposition.

According to Rozhnov & Rogoschik (1994), the longevity of the olfactory message (at least 60 days) seems to be similar to the length of time a spraint generally persists in the environment (8 weeks) (Mason & Macdonald, 1987).

Jenkins & Burrows (1980) contend that spraints are more likely to be found in wooded habitat and both sets of authors (Jenkins & Burrows / Mason & Macdonald) state that those deposited in sheltered positions can persist for up to a year. Otters are also known deposit spraints under bridges (Kruuk, 1992) which would also help to conserve the olfactory message.

Road kill and manipulation of otter movements

Road casualties have long been identified as the predominant cause of non-natural otter mortality (Green 1991; Strachan & Jefferies, 1996; Liles & Colley, 2000). Increases in otter road mortality recorded in Britain since 1983 have been more rapid than increases in other causes of otter death (Philcox et al, 1999). This may be partially due to heightened interest in road casualty and carcass documenting and recording.

With inevitable increases in roads, road traffic and (hopefully) otter numbers/distribution the problem of otter road mortality will remain. Otter friendly design of new roads and watercourse crossings plus modification of those already in existence would seem the bare minimum action necessary. The use of attractants and/or deterrents, developed from our understanding of odours might also allow us to begin manipulating otter behaviour in already established road blackspots.

Previous studies

Trowbridge (1983) investigated a possible mechanism for the ability shown by her test otter to differentiate between spraints from different individuals and to distinguish several spraints from a single individual. Volatile chemicals were extracted from otter spraints and then analysed using Gas Liquid Chromatography. The resultant data was subjected to extensive statistical analysis. The spraints were found to contain a highly complex mixture of common compounds. 98 peaks eluted at exactly the same retention time on chromatograms from all individuals. Although, they are not necessarily the same structures, the similarity between chromatograms implies that, rather than individual scent marks having components unique to an individual, the components are the same for all individuals, with their relative proportions providing the individual 'fingerprint'.

Trowbridge (1983) also showed that some compounds are specific to an individual otter. Although, they were not necessarily the compounds present in the largest concentrations, these chemicals would almost certainly contribute to the individuality of a scent mark.

There are many other reports of complex chemical profiles found in other mammal scent material:

Reindeer, *Rangifer tarandus* – Andersson et al (1975); Small Indian Mongoose, *Herpestes auropunctatus* – Gorman et al (1974); mink – Brinck et al (1978); European Moles, *Talpa europaea* – Khazanehdari et al (1996); Rabbits, *Oryctolagus cuniculus* – Goodrich & Mykytowycz (1972); African Mongoose – Hefetz et al (1984).

Many of the compounds present in these profiles were found to exist in varying relative concentrations. However, none of the differences were found to be significantly different.

Trowbridge's work, even by her own admission only offers a *possible* mechanism by which individual recognition could occur through olfactory means. The extraction procedure employed was not ideal. Diethyl ether (the solvent) may not have been suitable for dissolving *all* the volatiles involved in individuality (Trowbridge, 1983). Additionally, many volatiles were

probably lost during evaporation/concentration of the samples. A more preferable technique would involve headspace sampling, as suggested by Karlesen (1972) and Andersson et al (1975). There may even be levels of certain chemicals that are at such low levels that not even modern day techniques and apparatus are able to capture and detect them. These odours can only be analysed as far as present day technology will allow, and even then it is possible that the techniques and equipment would not measure up to the sensitivity of the animal itself.

Trowbridge (1983) demonstrated that otters have the ability to distinguish the spraint odour of different individuals, irrespective of sex or level of previous experience. Additionally, all spraints from a particular individual can be recognised and associated. This is possible even though the test animals were consuming identical diets. Therefore, individual recognition cannot rely solely upon digestive by-products (although this may contribute). The spraint odour profile comprises a complex mix of volatile chemicals, a selection of which vary in their relative concentrations between individuals. The odour profile is stable over time in spraints from the same individual (Trowbridge, 1983).

It is possible that subtle variations in the odour profile provide an animal with an individual scent. Some of the compounds found in other mammalian odours have been isolated, identified, and shown to possess unique odours (van Dorp et al, 1973). Mixtures of these individual chemicals will produce an almost infinite variety of aromas.

A limited number of studies have been carried out the anal scent sacs of the Eurasian otter, particularly as regards elucidation of secretion chemical constituents (Gorman et al, 1978; Jenkins et al, 1981; Bradshaw et al, 2001)

Gorman et al (1978) carried out a study into the anal scent sacs of the European otter, *Lutra lutra*. The anal sac secretion was examined using 3 methods:

- Thin Layer Chromatography (TLC) – a similar pattern for all individuals was observed with the following chemical types:
    - monoglycerides
    - steroids
    - fatty acids
    - triglycerides
    - sterol esters
- Gas-Liquid Chromatography (GLC) – Deposits had a typical ‘ottery’ smell, except for the white deposits seen in captive animals and the wild deposits. NB the white, green and

brown deposits all had identical constituents, but the major peak seen in the green/brown samples was seen at a much lower levels in the white samples. Gorman et al (1978) suggested that this major component might possibly signal 'otter'. Secretion from the two otters used could be differentiated between when fresh, however these differences were not stable with time.

- Gradient acrylamide gel electrophoresis (GAGE) – this gave a resolution of the anal sac secretion protein components and showed clear individual specific patterns that remained stable for at least 3 months. Gorman et al (1978) thought this to be a possible method for following wild otter movements, however, they considered that confusion due to protein bands from associated faeces, urine, sperm, and the substrate might cause serious problems.

It was concluded that GLC profiles were too labile with time to be used in monitoring of wild individuals. This seems to be at odds with work done in 1983 by Trowbridge where it was demonstrated that spraint odour profiles do not vary greatly with time (10 spraints were taken over a 25-day and analysed). They also state that as various agents were likely to contaminate anal secretions in the wild, that electrophoresis would only be of use for monitoring wild otters if fresh, uncontaminated secretions were used for analyses.

Jenkins et al (1981) attempted to improve on the GAGE technique used by Gorman et al (1978) by using Two-Dimensional Immuno-Electrophoresis (TDIE) to overcome the problems of contamination. Unfortunately, although it was possible to raise antibodies against otter anal sac secretions, these antibodies were found to be specific to otters in general rather than to individuals. Therefore the TDIE technique would be of no use in distinguishing between individual otters for monitoring purposes.

Bradshaw et al (2001) analysed the contents of scent glands from 25 otter corpses (19 males; 15 mature, 4 immature and 6 females, 5 mature, 1 immature) using purge and trap Gas Chromatography – Mass Spectrometry. In all, 47 volatile organic compounds were identified with GC-MS alone and these were classified as alkylated furans, alcohols, aldehydes, acid alkyl esters (up to C_6) and organic sulphur compounds. Significant differences between the secretion volumes found in the anal sacs of the same animal were seen. This may be either to one anal sac being more efficient at secretion production or one sac having been voided prior to death. In terms of their chemical profile the secretion from each sac was identical.

13 main chemical constituents were confirmed using standards: 2-methyl-propanal; 3-methyl-butanal; 2-methyl-butanal; 2-ethyl-furan; dimethyl disulphide; 1-pentanol; hexanal; 2-methyl-butanoic acid ethyl ester; 3-methyl-butanoic acid ethyl ester; 5-methyl-hexanal; benzaldehyde (the most abundant compound identified); dimethyl trisulphide and 2-pentyl-furan.

Immature otters were found to have significantly higher levels of *3-methyl-butanoic acid ethyl ester* than mature otters. No other significant differences for other chemicals were found between age classes or sexes.

Principal Component Analysis carried out on data from the main 13 identified compounds demonstrated female anal sac secretion profiles to be clustered within a broader male distribution.

Only one study has looked at spraint odour profiles (Trowbridge, 1983) but no elucidation of the chemical constituents was undertaken.

AIMS AND OBJECTIVES

- To investigate the use of SPME in the extraction of otter spraint odour chemicals.
- To develop a technique for ageing spraints, possibly as a tool for monitoring purposes, by mapping the chemical changes in an otter spraint in the hours following deposition.
- To determine whether spraint odour (extracted with SPME) can be used to identify individual European otters using a so-called otter fingerprint. Spraints from other otter species will also be analysed as a comparison.
- To investigate the fatty acid content of both European otter spraints and anal sac secretion in order to identify any individual or sexual differences. Again, spraints from other otter species will also be analysed as a comparison.

CHAPTER 2

USE OF SOLID PHASE MICRO-EXTRACTION (SPME) IN THE EXTRACTION OF OTTER SPRAINT VOLATILES

INTRODUCTION

Extraction of *relevant* volatile chemicals in the scent mark odour profiles is crucial in the understanding of mammalian chemical communication. The first identification of a mammalian pheromone was achieved in the mid-eighties (house mouse – Novotny et al, 1985; Jemiolo et al, 1986). Since this time, huge advances have been made in bio-analytical techniques, e.g., combined capillary gas chromatography/mass spectrometry and liquid chromatography / mass spectrometry (Novotny & Soini, 2007). Despite these methodological improvements few compounds or mixtures of compounds have been shown definitively to be mammalian pheromones (Burger, 2005) and relatively little is known about individual chemiosignals and the biochemical processes involved in their perception (Novotny & Soini, 2007).

Current analytical and separation techniques are able to resolve almost any type of complex mixture, with detection limits down to the femtogram range (Vas & Vékey, 2004). Sample preparation techniques involving the use of solvents (liquid-liquid extraction - LLE) are multi-step (separation from matrix / concentration / fractionation / derivatisation), labour intensive, time consuming procedures (Mills & Walker, 2000; Vas & Vékey, 2004; Kataoka, 2005). Most analytical instruments are not equipped to process sample matrices directly and this results >80% of analysis time being used up in the collection and preparation of samples (Vas & Vékey, 2004). Errors and losses can occur during every step, particularly when evaporation of volatile compounds is carried out. In addition, solvent waste must be disposed of, which adds extra expense to the procedure, has an environmental impact as well as creating health and safety problems for laboratory staff (Mills & Walker, 2000; Vas & Vékey, 2004; Kataoka, 2005).

Many of the limitations in LLE methodologies have been reduced with the use of Solid Phase Extraction, SPE (Vas & Vékey, 2004). For example, SPE requires less (although still significant) amounts of solvent. However, it is still a time consuming, multi-step procedure that sometimes uses a concentration step resulting in a loss of volatiles (Mills & Walker, 2000; Vas & Vékey, 2004; Kataoka, 2005). Additionally, clotting, channelling and percolation are typical problems of the SPE method (Vas & Vékey, 2004; Kataoka, 2005). Automated systems for LLE and SPE, although available, are

complex and as a result these extraction methods are normally performed off-line (Vas & Vékey, 2004; Kataoka, 2005).

From the perspective of mammalian scent mark odour extraction one fundamental disadvantage of these methods is that they do not provide a true representation of the odour profiles that scent marking materials possess. Firstly, the volatiles are not extracted from the air, i.e., the way they would naturally be conveyed between individuals (via a deposited spraint). Secondly when exposed to solvents, chemicals are extracted from the material which possibly would not appear in the natural scent.

A relatively recent and successful technique for sample preparation is solid-phase microextraction (SPME). Invented in 1989 (Belardi & Pawlisyzn, 1989; Arthur & Pawlisyzn, 1990) it attempted to solve the limitations found with LLE and SPE (Vas & Vékey, 2004; Kataoka, 2005). SPME combines analyte extraction, pre-concentration and sample introduction into a single (solvent free) step and can extract analytes from solid, liquid or gaseous samples. A silica fibre, 1cm in length is coated with a polymer and bonded to a stainless steel plunger. This is then housed in a holder that appears like a modified microlitre syringe. Fibres can be quickly and easily identified (through a window in the barrel of the holder) and interchanged; facilitated by a hand-tight needle hub assembly.

Normally the type of fibre required is chosen on the basis of the molecular weights and polarity of the compounds of interest (see Table 1).

The fibre is retracted into the needle, which is then pushed through the sample vial septum. The plunger is then depressed in order to expose the fibre to the sample or the headspace above it. The fibre remains exposed to the sample until equilibrium is reached. Analytes adsorb to the fibre coating becoming concentrated and the extraction process is based on a similar principle to that of chromatography; gas-liquid or liquid-liquid partitioning (Vas & Vékey, 2004).

Table 1. SPME fibre types

Type of Compound	Colour code	Type of Fibre
Low molecular weight / Volatile compounds	Red	100µm polydimethylsiloxane (PDMS) coated fibre
Volatiles, Amines and Nitroaromatic compounds	Blue	65µm polydimethylsiloxane /divinylbenzene (PDMS/DVB) coated fibre
Polar Semivolatiles	White	85µm polyacrylate coated fibre
Non-polar, high molecular weight compounds	Green	7µm polymethylsiloxane coated fibre

When adsorption equilibrium is reached the fibre is again retracted and the needle withdrawn from the sample vial. The final step of the process involves introduction of the fibre to the gas chromatograph injector, where the analytes that have been adsorbed are then thermally desorbed and delivered onto the GC column (or eluted by the mobile phase in the case of HPLC). See Figure 1 for a graphical representation of the extraction process.

The SPME method reduces sample preparation time, lowers disposal costs and can improve detection limits (Vas & Vékey, 2004; Kataoka, 2005).

Since SPME was invented by Pawlisyzn and his co-workers (Belardi & Pawlisyzn, 1989; Arthur & Pawlisyzn, 1990) there has been a huge increase in the number of applications for the technique. Early uses focussed on the analysis of pollutants in environmental matrices (Pawlisyzn, 1997; Eisert & Pawlisyzn, 1997). Uses then expanded to include the measurement of volatile and semi-volatile substances in flavourings, beverages, foodstuffs, pharmaceutical products and forensic samples.

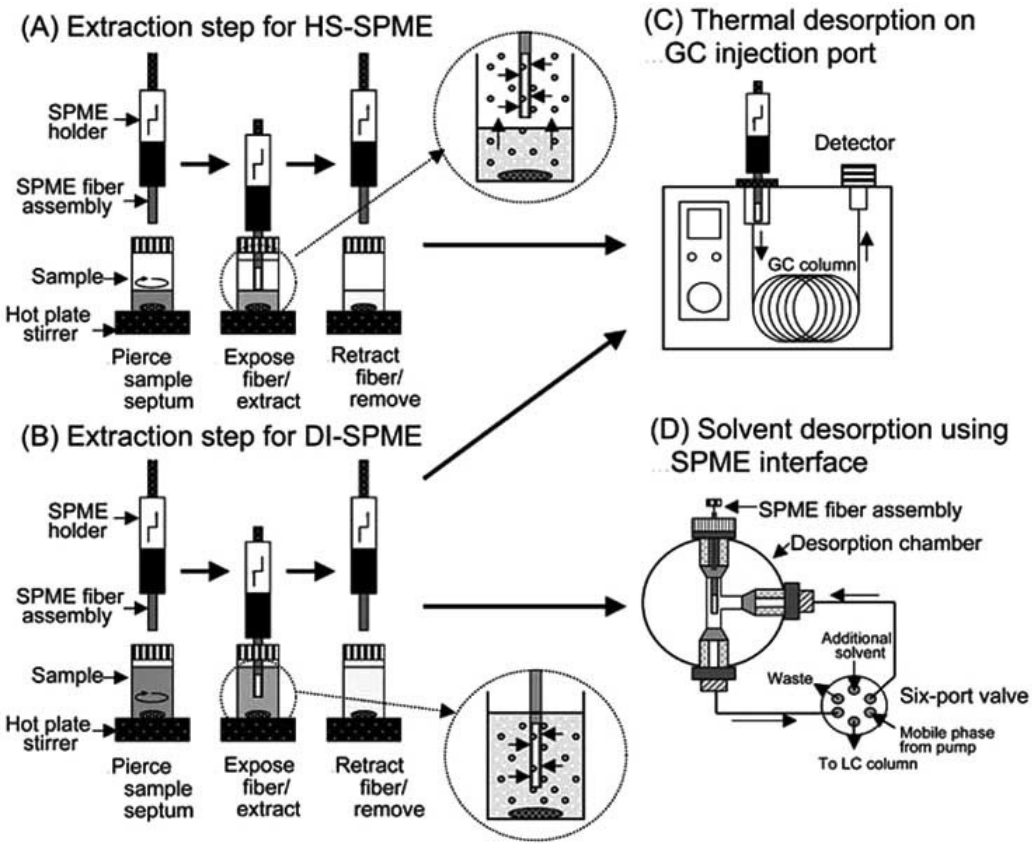


Figure 1. Extraction process by Headspace (HS) and Direct Immersion (DI) fibre SPME, and desorption systems for GC and HPLC analyses (taken from Katoaka, 2005)

Then, more recently, headspace SPME has been used with clinical and toxicological samples (Chiarotti & Marsili, 1994). Examples include: urine (Yashiki et al, 1995; Guidotti et al, 1999); blood (Seno et al, 1996; Namera et al, 2000; Liu et al, 1999); faeces (Mills et al, 1999; Mills & Walker, 2000); breast milk (Rohrig & Meisch, 2000; DeBruin et al, 1998); hair (Lucas et al, 2000; Strano-Rossi & Chiarotti, 1999); breath (Grote & Pawlisyzn, 1997); saliva (Hall et al, 1998; Lucas et al, 2000) and axillary sweat (Curran et al, 2005).

The majority of studies into anal scent marking material to date have utilised some form of solvent extraction: n-pentane (Brinck et al, 1978); dichloroethane (Gorman et al, 1978); di-ethyl ether (Trowbridge, 1983; Crump, 1980a) methylene chloride (Brinck et al, 1983; Hefetz, et al, 1984): headspace analysis with activated charcoal then extraction using dichloromethane (Khazanehdari et al, 1996).

Solid phase micro extraction (SPME) has not been used to any great degree for headspace analysis of mammalian scent mark volatiles. SPME is very useful in qualitative analysis, however it must used carefully when comparing the quantitative

composition of samples with large variation in water and/or lipid content. Water will compete with the fibre for polar constituents producing a distortion in quantitative results (Burger, 2005). If analytes are present in low concentration in, e.g., aqueous media or waxy matrices, adsorption via SPME could be very poor (Burger, 2005). SPME can be used, however, to investigate changes in the rate of volatile release from scent marks (Burger et al, 1997).

There are a limited number of studies that have used SPME to investigate mammalian scent marking material. Examples include: subcaudal secretion of the owl monkey, *Aotus nancymaae* (Macdonald et al, 2007); anal gland secretion, vaginal secretion and urine of the giant panda, *Ailuropoda melanoleuca* (Hagey & Macdonald, 2003); anal gland secretion of the spotted hyena, *Crocuta crocuta* (Burgener et al, 2007).

No studies (to date) have used headspace SPME to investigate *otter* spraint volatiles. As SPME does not use solvents there is no masking of early eluting compounds with a solvent peak. Also they extract chemicals from the air in headspace analysis, so the procedure is more akin to the way in which otters detect spraint volatiles in their natural situation. This part of the study aims to optimise the SPME technique for use with otter spraints. There are a number of considerations including fibre selection, extraction optimisation and desorption optimisation.

Fibre selection

Generally speaking, the extraction of volatiles via SPME is most effective when the polarity of the fibre matches that of the analyte (Mills & Walker, 2000). Similarly, analytes require columns of the similar polarity for analysis in Gas Chromatography (Mani, 1999).

Extraction optimisation

The amount of analyte extracted depends on:

- Polarity and thickness of the stationary phase (Mills & Walker, 2000)
- Extraction time and concentration of analytes in sample (Kataoka et al, 2000; Pawliszyn, 1999)
- Agitation (Kataoka et al, 2000; Pawliszyn, 1999)

- Addition of salt (Kataoka et al, 2000; Pawliszyn, 1999)
- Changing pH (Kataoka et al 2000; Pawliszyn, 1999)
- Increasing temperature (Kataoka et al, 2000; Pawliszyn, 1999)
- Vial size and sample volume (Kataoka et al, 2000; Pawliszyn, 1999)

The rationale in using SPME in this research is to attempt to extract those volatiles from a spraint that an otter would extract in as similar way as possible to the animal itself. Extraction using solvents requires agitation of the sample which results in chemicals from the body of the spraint being extracted; this is undesirable. Using headspace SPME in contrast, only those volatiles that are released from the surface of the spraint are extracted. In a similar way, some of the methods for optimising the amount of analyte extracted from the spraint (i.e., agitation, addition of salt, changing pH) were deemed unsuitable for use spraints if the method was to extract chemicals in a similar way to the animal. All other methods were used in the optimisation process:

- Extraction time (Kataoka et al, 2000; Pawliszyn, 1999)
- Increasing temperature (Kataoka et al, 2000; Pawliszyn, 1999)
- Vial size and sample volume (Kataoka et al 2000; Pawliszyn, 1999)

Optimisation of desorption

Efficient analyte desorption in the GC port is dependent upon:

- Fibre coating thickness
- Injection depth
- Injection temperature
- Exposure time
- Narrow bore GC injection insert

Splitless mode operated

MATERIALS AND METHODS

Instrumentation, technical specifications and equipment

Gas chromatograph

All chemical analyses were performed on a Gas Chromatograph (Hewlett Packard 5890 Series II). The carrier gas was helium at 2mL/min, constant flow. Flame Ionisation Detector (FID).

GC column

Column type, SGE, (Analytical Science) - Bonded Phase (BP1) non-polar; column dimensions, 25m x 0.33mm; temperature limits; -60 to 340°C; material, fused silica; film thickness, 0.25µm; I.D., 0.22mm.

Inlet liner

0.75mm Inner Diameter SPME inlet liner (Supelco, Bellefonte, PA)

Temperature programmes

i. The chromatograph was run in a splitless mode (closed 2 min) and the following additional parameters: injector temperature 250ºC; detection temperature 300ºC; initial oven temperature 35ºC for 2 min; this was then increased by 5ºC/min up to 170ºC, this temperature was maintained for 2 min; then there was a further increase of 15ºC/min up to 270ºC, again maintained for 2 min; fibre desorption time was 2 min.

ii. The chromatograph was run for both the injection and the SPME extraction in a splitless mode (closed 2 min) and the following additional parameters: injector temperature 270ºC; detection temperature 300ºC; initial oven temperature 35ºC for 2 min; this was then increased by 5ºC/min up to 170ºC, this temperature was maintained for 2 min; then there was a further increase of 15ºC/min up to 270ºC, again maintained for 2 min; fibre desorption time was 5 min.

Sampling equipment (all supplied by Supelco, Bellefonte, PA)

Table 2. Sampling equipment

Fibre holder	standard fibre holder for manual sampling (see Figure 1.).	
Fibres used	Red	100µm polydimethylsiloxane (PDMS)
	Blue	65µm polydimethylsiloxane/divinylbenzene (PDMS/DVB)
	White	85µm polyacrylate
	Green	7µm polymethylsiloxane
SPME sampling stand	holds 8 vials while supporting fibre holder for consistent fibre depth	
Vials	Amber vial, screw top 4mL with hole cap, PTFE/Silicone Septa	

Fibre conditioning

All fibres require 'conditioning' (cleaning) before any analysis can be carried out. Each fibre has its own temperature and conditioning recommendations for GC use (see Table 3).

The conditioning effectively desorbs chemicals from the fibre leaving it clean and ready to be used in analyses. A new fibre, although unused will give off extraneous peaks primarily from the adhesives used to attach the fibres. Conditioning is necessary between each analysis. A blank run was therefore required, after conditioning and before exposure to spraints to ensure the fibre was clean.

Table 3. SPME fibre operating temperatures.

Colour code	Type of Fibre	Max Temp	Operating Temp	Conditioning Temp	Time (hrs)
Red	100µm polydimethylsiloxane (PDMS)	280ºC	200-270ºC	250ºC	1
Blue	65µm polydimethylsiloxane /divinylbenzene (PDMS/DVB)	270ºC	200-270ºC	260ºC	0.5
White	85µm polyacrylate	320ºC	220-310ºC	300ºC	2
Green	7µm polymethylsiloxane	340ºC	220-320ºC	320ºC	2-4

Fibre selection

The most appropriate SPME fibre for sampling spraint associated odour components was selected using a captive adult male spraint sample acquired from the Tamar sanctuary, North Petherwin, Cornwall.

i. A sub-sample of spraint material (1g) was placed in a 4mL airtight vial.

ii. The SPME fibre was exposed to the headspace of the sub-sample in an upright position for exactly 30mins at room temperature.

iii. The adsorbed odour components were then analysed using Gas Chromatography (temperature programme i).

iv. A replicate analysis was performed.

This procedure was carried out for each of the 4 different fibres (green; white; red; blue – see Table 3). The resultant chromatograms were compared in order to select the most appropriate fibre, i.e. the fibre that extracted the largest number of odour chemicals from the spraint material.

Comparison of SPME to direct injection

A standard chemical array: 1mL Toxic Organics Mix 1-A (TOM1A), Supelco (Bellefonte, PA) containing 2000µg/ml of each of 8 different volatile organic components in methanol (n-heptane; 1-heptane; benzene; toluene; ethylbenzene; p-xylene; isopropylbenzene; o-xylene) was used to compare direct injection into the GC with headspace analysis using SPME.

The mixture was split into 200µL aliquots and stored in airtight vials.

i. A 1µL aliquot of TOM1A was directly injected into the Gas Chromatograph (see programme below). 4 replicates were performed.

ii. 200µL of standard were placed in a 4mL airtight vial.

iii. A blue (65µm polydimethylsiloxane /divinylbenzene) SPME fibre was exposed to the headspace of the standard in an upright position for exactly 30mins at room temperature.

iv. The adsorbed standard hydrocarbons were then analysed using Gas Chromatography (temperature programme i). 5 replicates were performed.

Area under the peaks from all resultant chromatograms were normalised, and mean values calculated for the 4 replicates. Direct injection and SPME headspace extraction were then compared for each substance.

Four standards (benzene; 1-heptene; n-heptane; toluene) were purchased and injected separately in order to link the peaks obtained on the GC to the chemicals contained in TOM1A. All were dissolved (1:100) in methanol; 1µL was injected (temperature programme i).

Extraction and Desorption optimisation

Extraction time

The effects of different SPME fibre exposure times to the headspace of TOM1A was investigated.

i. 200µL of standard were placed in a 4mL airtight vial.

ii. A red (100µm polydimethylsiloxane) SPME fibre was exposed to the headspace of the standard in an upright position for exactly 1, 5, 10, 20 and 40 mins at room temperature.

iii. The adsorbed standard hydrocarbons were then analysed using Gas Chromatography (temperature programme i).

NB This procedure was repeated with a blue (65µm polydimethylsiloxane /divinylbenzene) fibre.

Extraction temperature / Sample:Headspace ratio / Desorption temperature

Variation in the SPME method was investigated using an adult male otter sample from the Bowes Otter Sanctuary and also using a hydrocarbon standard (C_{11} – C_{15}, dissolved 1:100 in methanol). See Table 4 for summary.

The area (≡concentration) under each of the peaks produced for each of the resultant chromatographs from the above analyses were normalised, standard deviations and arithmetic means were generated and the coefficient of variation was then calculated for each peak value.

Table 4. Comparison of the original and optimised extraction / sampling procedures for spraint material and hydrocarbon standard using a red (100µm polydimethylsiloxane) SPME fibre and direct injection.

		SPME		**Direct Injection**
		Spraint material	**Hydrocarbon standard (C_{11}-C_{15})**	**Hydrocarbon standard (C_{11}-C_{15})**
Original procedure	***Sample volume / ratio to headspace in 4mL vial***	1mL / 1:3	200µL / 1:19	1µL
	Fibre exposure time	20 mins	20 mins	
	Extraction temperature	Ambient temperature in lab	Ambient temperature in lab	
	GC temperature programme	i: injector temperature - 250°C; fibre desorption time: 2mins	i: injector temperature - 250°C; fibre desorption time: 2mins	i: injector temperature - 250°C; fibre desorption time: 2mins
Optimised procedure	***Sample volume / ratio to headspace in 4 mL vial***	2mL / 1:1	200µL / 1:19	
	Fibre exposure time	20 mins	20 mins	
	Extraction temperature	23°C in incubator	23°C in incubator	
	GC temperature programme	ii: injector temperature - 270°C; fibre desorption time: 5mins	ii: injector temperature - 270°C; fibre desorption time: 5mins	

- 4 replicates were carried out for each procedure
- The *optimised* SPME procedure was repeated using a blue (65µm polydimethylsiloxane /divinylbenzene) fibre

RESULTS

Fibre selection

Figures 2 - 5 show example chromatograms obtained with each of the 4 different fibre types (green; white; red; blue – see Table 3). These chromatograms were compared to select the fibre that extracted the largest number of odour chemicals from the spraint material.

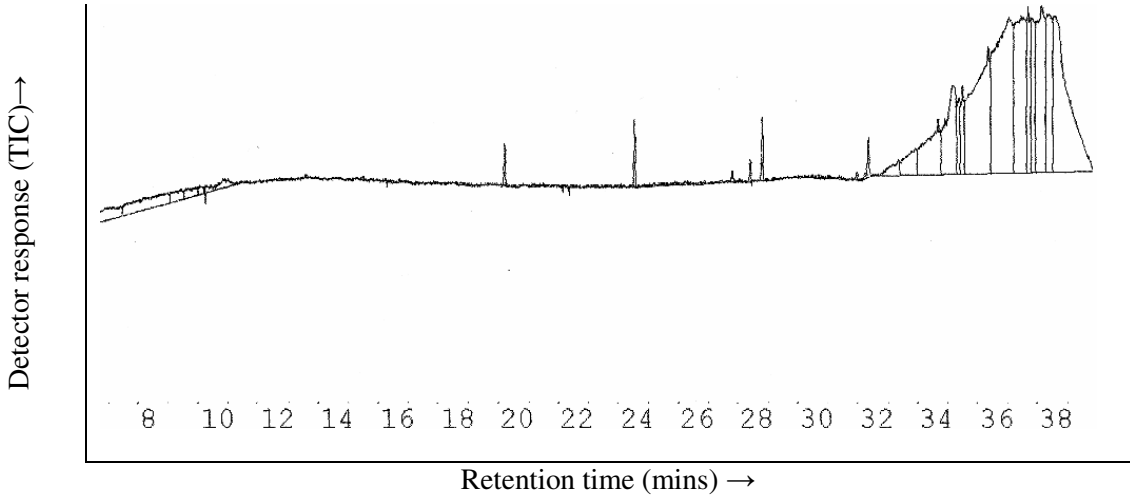


Figure 2. Gas chromatogram of spraint odour using the green (7µm polymethylsiloxane) fibre.

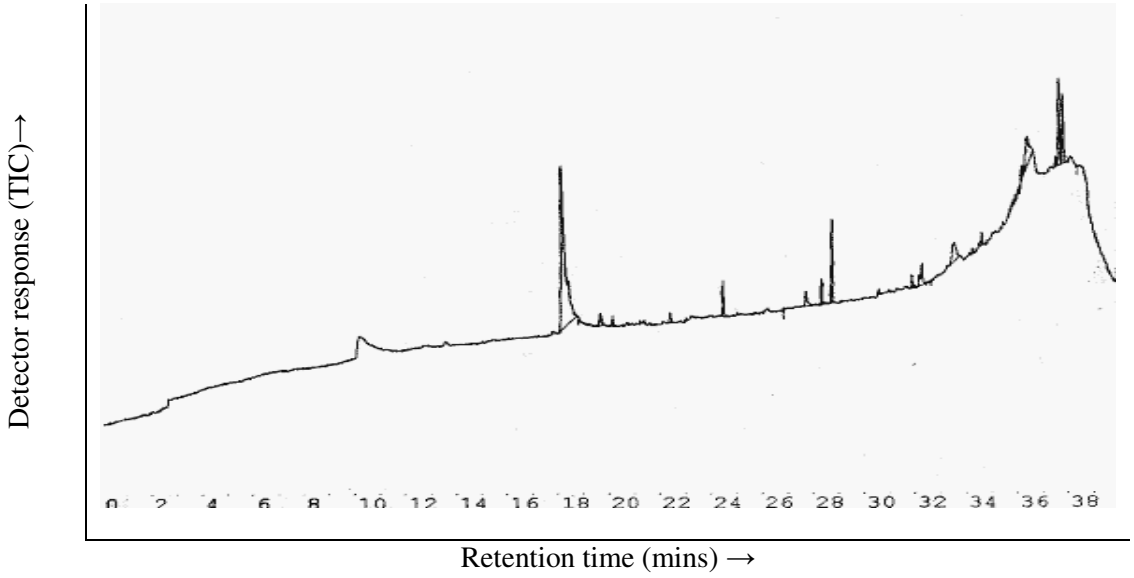


Figure 3. Gas chromatogram of spraint odour using the white (85µm polyacrylate) fibre

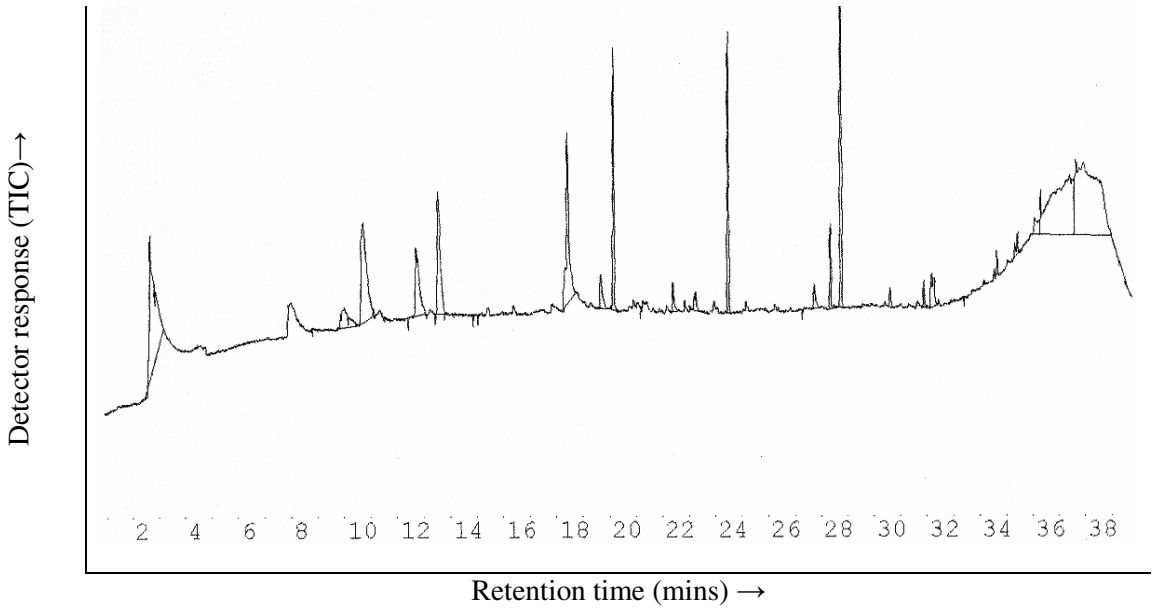


Figure 4. Gas chromatogram of spraint odour using the red (100µm polydimethylsiloxane) fibre

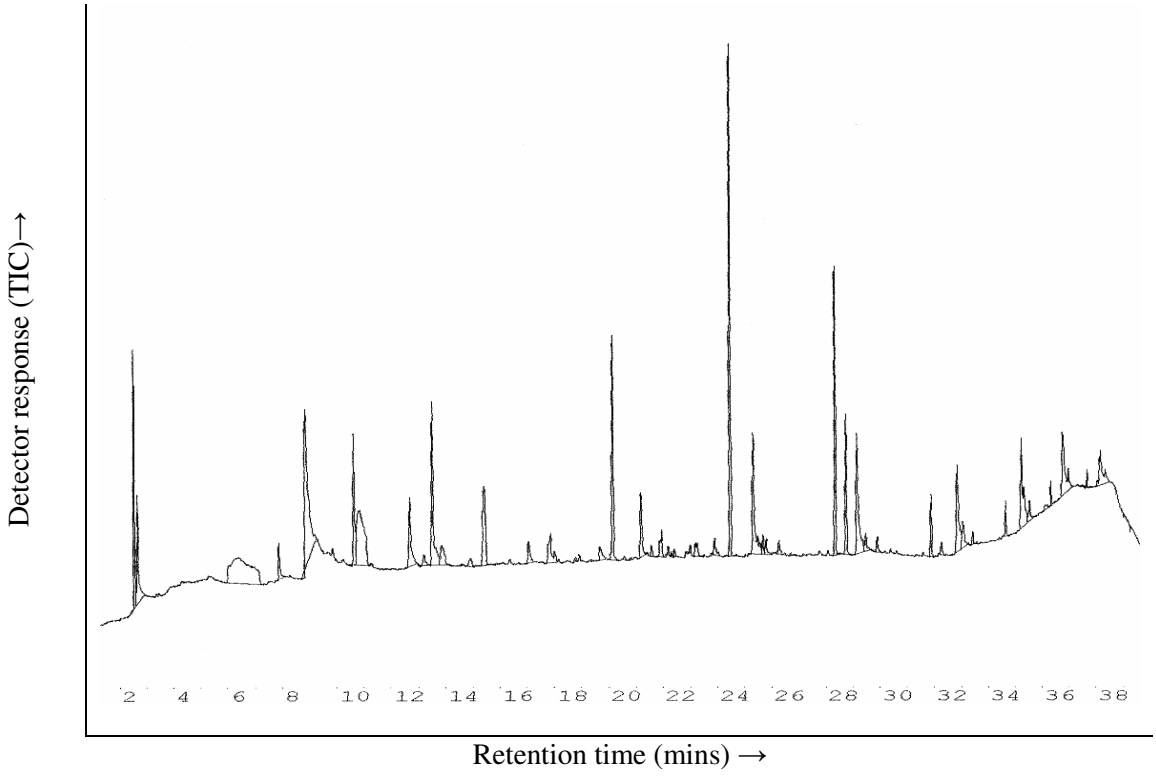


Figure 5. Gas chromatogram of spraint odour using the blue (65µm polydimethylsiloxane /divinylbenzene) fibre.

Figures 4 and 5 show that the red and blue fibres have the biggest capacity for adsorbing otter spraint odour compounds in comparison with the green and white fibres.

Comparison of SPME to direct injection

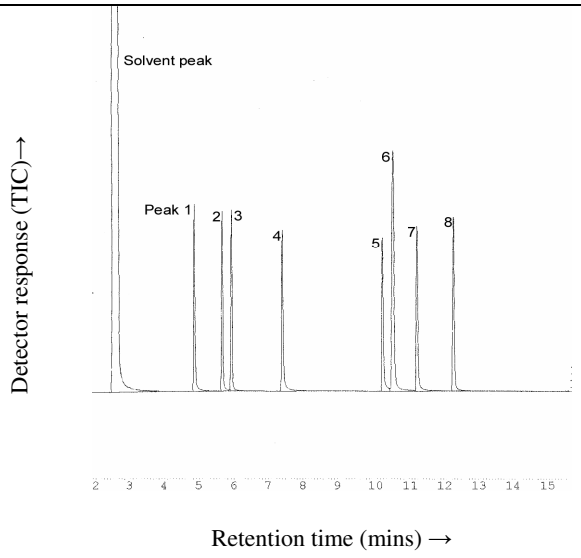


Figure 6. Example chromatograph of direct injection of 1µL of TOM1A

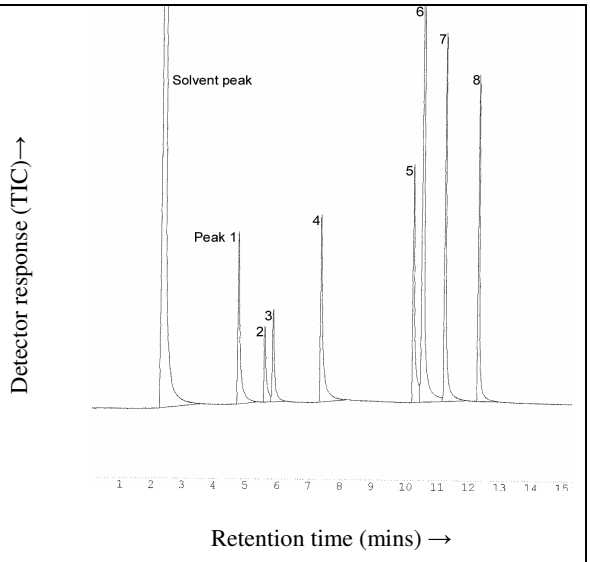


Figure 7. Example chromatograph of exposure of blue fibre to 200µL of TOM1A for 20mins at room temp

Table 5. Comparison of direct injection with SPME.

Peak no.	Retention time	*Mean* Conc. (area under peak) x 10^6	
		Direct injection	SPME
1	4:59	7.75	1.61
2	5:48	6.54	0.64
3	6:04	6.70	0.79
4	7:33	7.73	1.78
5	10:27	7.54	1.81
6	10:45	1.65	4.99
7	11:27	8.32	2.69
8	12:31	8.17	2.14

Figures 6 and 7 indicate that direct injection of Toxic Organics Mix 1-A provides very different concentrations of the 8 volatiles, when compared with SPME headspace analysis.

Mean concentration figures from 4 identical runs for each chemical are seen in Table 5. These figures confirm that direct injection of TOM 1-A delivers higher concentrations of the 8 chemicals when compared to the SPME headspace analysis technique.

Table 6 presents the retention times of the injected standards and matches them to retention times of peaks 1 – 4 in the chromatogram. This is a standard method of positively identifying unknown chemicals. After identification of substances using GC/MS this can be used as a final check.

Table 6. Identification of individual peaks using standards.

Peak	Retention times (mins)		Chemical
	TOM 1-A	Individual standard	
1	4.59	5.01	benzene
2	5.48	5.51	1-heptene
3	6.04	6.06	n-heptane
4	7.33	7.34	toluene

Extraction and desorption optimisation

Extraction time

Figure 8 shows the positive relationship between increasing fibre exposure time and substance concentration when using the red (100µm polydimethylsiloxane) fibre and how the concentration reaches a plateau after approximately 20 mins.

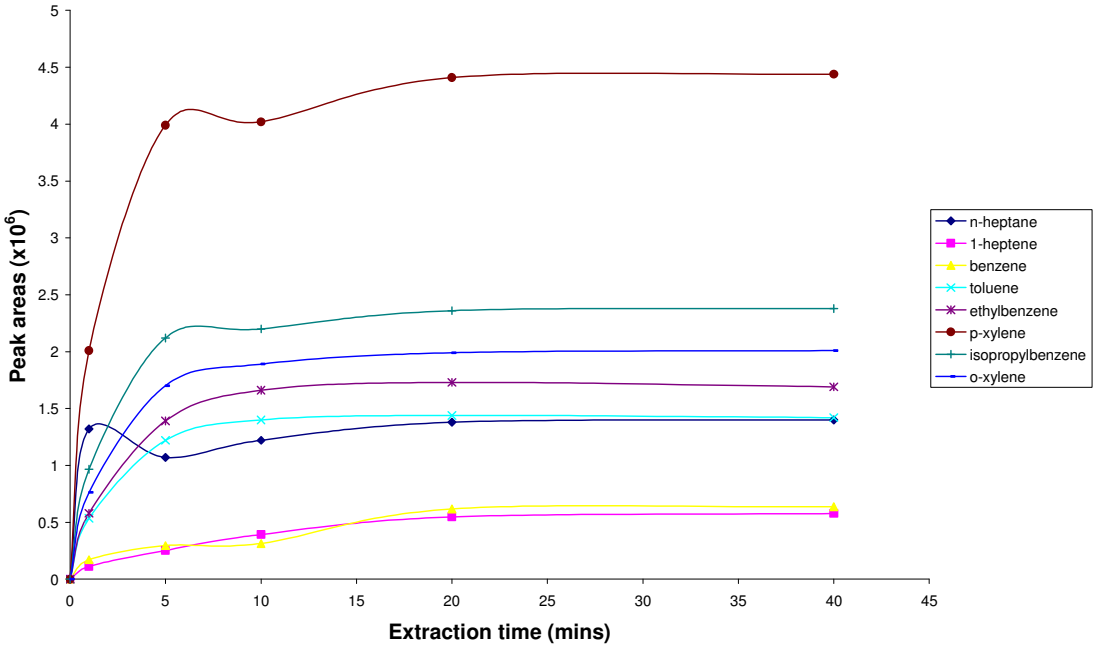


Figure 8. Relationship between increasing fibre exposure time and substance concentration when using the red (100µm polydimethylsiloxane) fibre.

Figure 9 shows the positive relationship between increasing fibre exposure time and substance concentration when using the blue (65µm polydimethylsiloxane /divinylbenzene) fibre and how the concentration reaches a plateau again after approximately 20 mins.

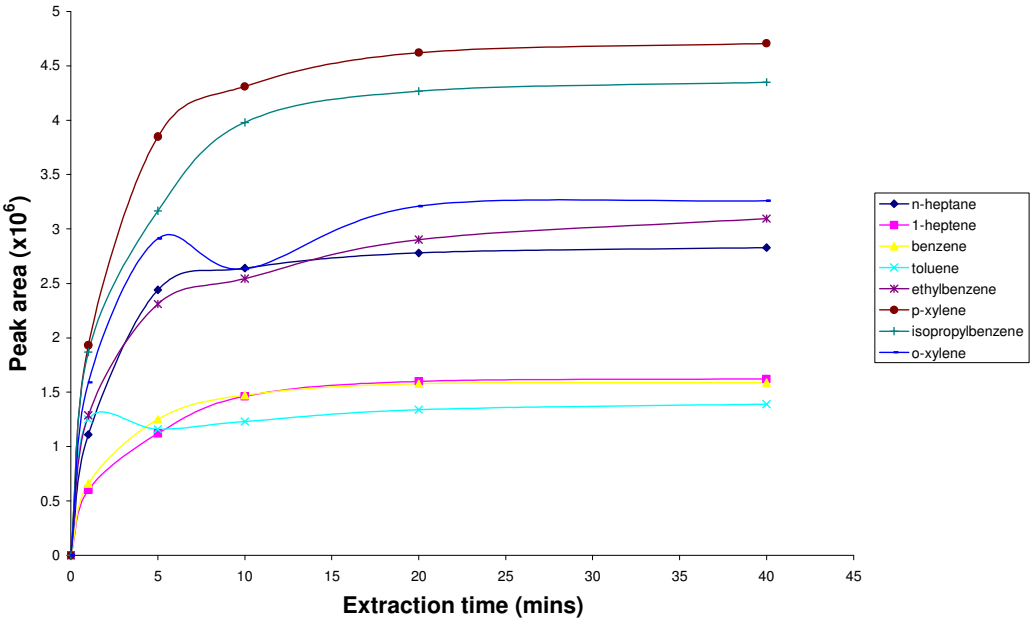


Figure 9. Relationship between increasing fibre exposure time and substance concentration when using the blue (65µm polydimethylsiloxane /divinylbenzene) fibre.

Extraction temperature / Sample: Headspace ratio / Desorption temperature

Original procedure:

SPME extraction of spraint material using a red (100µm polydimethylsiloxane) fibre

Table 7. Spraint extraction using a red (100µm polydimethylsiloxane) fibre prior to method optimisation (normalised data)

Peak no	Replicate extractions with red fibre					Mean	StDev	**CoVar (%)**
	A	B	C	D	E			
1	6.82	15.32	9.53	6.78	3.76	8.44	4.35	**51.56**
2	20.96	0.89	18.59	23.62	25.03	17.82	9.78	**54.89**
3	9.94	13.13	13.41	11.60	10.20	11.66	1.61	**13.80**
4	8.83	7.72	9.18	6.60	7.42	7.95	1.05	**13.26**
5	12.71	11.52	6.32	6.39	7.94	8.98	2.97	**33.04**
6	8.21	12.64	8.99	8.80	10.56	9.84	1.79	**18.19**
7	6.33	9.12	6.23	6.86	5.75	6.86	1.32	**19.28**
8	26.20	29.66	27.75	29.34	29.35	28.46	1.47	**5.16**

This initial test of the SPME method with spraint material produced a great deal of variation demonstrated by the Coefficient of Variation percentage figures seen in the far right hand column (Table 7 above). The extraction of some peaks varied more significantly than others, e.g, peak 1 showed a coefficient of variation of 51.56% which was approximately 10x the variation seen in peak 8 (5.16%).

SPME extraction of hydrocarbon standard using a red (100µm polydimethylsiloxane) fibre

Table 8. Standard hydrocarbon extraction using a red (100µm polydimethylsiloxane) prior to method optimisation (normalised data)

Hydrocarbon	Replicate extractions with red fibre					Mean	StDev	**CoVar (%)**
	A	B	C	D	E			
C_{11}	65.34	72.60	56.20	68.54	62.09	64.95	6.25	**9.63**
C_{12}	11.49	6.47	6.26	10.51	12.80	9.51	2.98	**31.34**
C_{13}	16.64	12.55	6.49	11.21	16.20	12.62	4.14	**32.80**
C_{14}	0.60	1.59	0.92	0.90	1.40	1.08	0.40	**37.37**
C_{15}	5.93	6.78	6.47	8.85	7.52	7.11	1.13	**15.86**

A similar test of the SPME method, this time with a hydrocarbon standard, also produced a great deal of variation, again demonstrated by the Coefficient of Variation percentage figures (Table 8 above). The extraction of some peaks varied more significantly than others, e.g, C_{14} showed a coefficient of variation of 37.37% which was approximately 4x the variation seen in C_{11} (9.63%).

Direct injection of hydrocarbon standard

Table 9. Direct injection (1µL aliquot) of hydrocarbon standards (normalised data)

Hydrocarbon	Replicate injections					Mean	StDev	**CoVar (%)**
	A	B	C	D	E			
C_{11}	22.80	23.80	21.01	20.99	21.55	22.03	1.23	**5.60**
C_{12}	22.39	21.68	19.94	19.59	21.21	20.96	1.18	**5.62**
C_{13}	21.11	20.14	19.94	20.45	20.36	20.40	0.44	**2.18**
C_{14}	17.86	17.89	19.83	19.70	20.61	19.18	1.24	**6.46**
C_{15}	15.98	18.61	19.29	19.27	16.27	17.88	1.63	**9.12**

A comparison of direct injection of the Hydrocarbon standard with extraction using SPME highlighted a marked difference between the two methods. The direct injection

method showed much less variation than the SPME method, demonstrated by consistently lower Coefficient of Variation figures (all were <10%, see Table 9).

Optimised procedure:

SPME extraction of spraint material using a red (100µm polydimethylsiloxane) fibre

Table 10. Spraint extraction using a red (100µm polydimethylsiloxane) *following* method optimisation (normalised data)

Peak no	Replicate extractions with red fibre					Mean	StDev	**CoVar (%)**
	A	B	C	D	E			
1	6.71	5.61	6.62	6.19	5.51	6.13	0.56	**9.09**
2	24.76	24.34	22.21	23.98	25.92	24.24	1.35	**5.57**
3	11.68	12.05	10.86	12.50	10.77	11.57	0.75	**6.46**
4	8.64	8.17	6.69	7.52	7.26	7.66	0.77	**10.04**
5	6.78	6.06	5.77	6.23	5.87	6.14	0.40	**6.49**
6	7.87	8.85	9.33	9.18	8.76	8.80	0.57	**6.46**
7	6.23	6.62	7.45	6.87	6.81	6.80	0.44	**6.53**
8	27.33	28.29	31.06	27.53	29.12	28.67	1.51	**5.28**

Adjustments to the SPME extraction / desorption method (namely: sample: headspace ratio changed from 1: 3 to 1: 1; extraction conditions changed from extraction in the lab at room temperature to extraction in an incubator at a constant 23°C; injector temperature changed from 250°C to 270°C; desorption time increased from 2 mins to 5 mins; introduction of 0.75mm ID inlet liner) resulted in much improved percentage Coefficient of Variation figures with only one peak (peak 4 – 10.04%) having a result >10%.

SPME extraction of hydrocarbon standard using a red (100µm polydimethylsiloxane) fibre

Table 11. Standard hydrocarbon extraction using a red (100µm polydimethylsiloxane) *following* method optimisation (normalised data)

Hydrocarbon	Replicate extractions with red fibre					Mean	STDev	**CoVar (%)**
	A	B	C	D	E			
C_{11}	56.37	54.15	54.48	55.41	58.26	55.73	1.66	**2.97**
C_{12}	8.48	8.48	8.84	9.14	9.34	8.85	0.39	**4.39**
C_{13}	26.31	27.61	26.87	25.98	24.20	26.19	1.27	**4.86**
C_{14}	1.53	1.80	1.90	1.88	1.70	1.76	0.15	**8.52**
C_{15}	7.31	7.96	7.91	7.60	6.50	7.46	0.60	**7.98**

A similar result was seen when these method adjustments were also applied to SPME extraction of the hydrocarbon standard mix, i.e., much improved percentage Coefficient of Variation figures. Here though, all peaks recorded figures of <10%.

SPME extraction of spraint material using a blue (65µm polydimethylsiloxane /divinylbenzene)fibre

Table 12. Spraint extraction using a blue (65µm polydimethylsiloxane /divinylbenzene) *following* method optimisation (normalised data)

Peak no	Replicate extractions with blue fibre					Mean	StDev	**CoVar (%)**
	A	B	C	D	E			
1	3.43	4.33	4.05	3.70	3.37	3.78	0.41	**10.88**
2	29.96	31.55	27.94	30.40	31.58	30.29	1.49	**4.92**
3	12.70	11.67	13.85	13.53	11.64	12.68	1.02	**8.08**
4	9.26	8.74	7.65	7.61	9.70	8.59	0.94	**10.99**
5	11.83	10.09	9.17	9.77	11.05	10.38	1.06	**10.20**
6	9.46	9.72	10.71	10.04	10.48	10.08	0.52	**5.15**
7	7.79	7.44	8.54	7.93	7.08	7.76	0.55	**7.06**
8	15.57	16.46	18.10	17.02	15.09	16.45	1.19	**7.24**

SPME extraction of hydrocarbon standard using a blue (65µm polydimethylsiloxane /divinylbenzene)fibre

Table 13. Standard hydrocarbon extraction using a blue (65µm polydimethylsiloxane /divinylbenzene) *following* method optimisation (normalised data)

Hydrocarbon	Replicate extractions with blue fibre					Mean	StDev	**CoVar (%)**
	A	B	C	D	E			
C11	56.37	54.13	54.48	55.41	58.26	55.73	1.66	**2.98**
C12	8.48	8.50	8.84	9.14	9.34	8.86	0.38	**4.31**
C13	26.31	27.6	26.87	25.98	24.20	26.19	1.27	**4.86**
C14	1.53	1.80	1.90	1.88	1.70	1.76	0.15	**8.52**
C15	7.31	7.96	7.91	7.60	6.50	7.46	0.59	**7.97**

The SPME method adjustments were also applied to extractions using the blue (65µm polydimethylsiloxane /divinylbenzene) fibre, both for spraint material and for the hydrocarbon standard. A similar result to that seen with the red fibre was achieved, with percentage Coefficient of Variation figures all <11% in the case of the spraint material (see Table 12) and all <9% in the case of the hydrocarbon standard (see Table 13).

Table 14 represents a comparison between the performance of the red and blue SPME fibres.

Table 14. Normalised (%) peak areas with 40 minute exposure

Compound	normalised (%) peak areas with 40 min exposure	
	red fibre	blue fibre
n-heptane	9.62	12.39
1-heptene	3.97	7.10
benzene	4.37	6.95
toluene	9.76	6.09
ethylbenzene	11.61	13.55
p-xylene	30.51	20.61
isopropylbenzene	16.35	19.04
o-xylene	13.81	14.27

DISCUSSION

Fibre selection

The results show the red (100µm polydimethylsiloxane) and blue (65µm polydimethylsiloxane /divinylbenzene) fibres to be the most effective for use in spraint headspace analysis as they extract far more odour chemicals than the other fibres. This is important as the more constituents of the odour profile that can be identified the more information regarding the nature of the spraint odour can be gained, which may potentially lead to a greater understanding of sprainting behaviour.

Comparison of SPME to direct injection

The results show direct injection of TOM 1-A delivers higher concentrations of chemicals when compared to SPME headspace analysis. This is a reasonable outcome as each substance has a different volatility and so concentrations in the headspace will not necessarily match those found in the body of the liquid mixture itself. Also, consideration has to given to the affinity of each different chemical to the SPME fibre.

Both techniques produce a solvent peak in this instance, however if extracting from organic material (e.g., spraints or scats) SPME offers an advantage over direct injection of substances in a solvent. Solvent peaks such as the one seen in Figures 8 and 9 (methanol) can obscure the presence of chemicals of a similar volatility. With SPME there is no such solvent peak (see Figures 4 and 5).

Extraction and desorption optimisation

Extraction time

The results show that increasing fibre exposure time is initially correlated with an increase in the amount of volatile chemicals adsorbing to the fibre. When the exposure time reaches 20 minutes the amount of chemicals adsorbing to the fibre levels off and this may indicate that a state of equilibrium, between the sample, the fibre coating and the gaseous headspace, has been achieved. NB until state of equilibrium is reached, analytes will migrate between all three phases (Pawliszyn, 1999). The equation seen in Figure 10 shows the linear relationship between the initial

concentration of analyte in the sample and the amount that is adsorbed by the fibre coating:

$$n = \frac{K_{fs} V_f C_0 V_s}{K_{fs} V_f + V_s}$$

Where:

n = mass of analyte adsorbed by coating

C_0 = initial concentration of analyte in sample

K_{fs} = partition coefficient for analyte between coating and sample matrix

V_f = volume of coating

V_s = volume of sample

Figure 10. Taken from Bulletin 923. Supleco website:
http://www.sigmaaldrich.com/Graphics/Supelco/objects/4600/4547.pdf

Figures 8 and 9 show the peak areas with a 20 minute or a 40 minute fibre exposure time to be similar. This is the case for both the red (100µm polydimethylsiloxane) and blue (65µm polydimethylsiloxane /divinylbenzene) fibres. Choosing a 20 minute exposure time would provide virtually identical results to a 40 minute exposure time and therefore 20 minutes was adopted as the optimum fibre exposure time.

Equilibration times should be carefully considered as it is possible that an extensive reduction in the curve gradient may be mistaken for the point of equilibrium (Pawliszyn, 1999). For example, in Figure 10 the slope of the curve for p-xylene drops away between the 5 and 10 minute exposure times, but then continues to rise thereafter.

When equilibrium is achieved, the amount of the analyte extracted by the fibre will be unaffected by small variations in the extraction time. However, if extraction times are taken from the steep part of the curve, then even tiny variations in extraction time will bring about substantial variation in the amounts extracted (Pawliszyn, 1999). Despite the fact that, in our study it seems equilibrium has been achieved with a 20 min exposure time, it must be borne in mind that this may not actually be the case and that the curve could continue to rise with exposure times longer than 40 minutes. In order

to produce good accuracy and precision with SPME, 'consistency' in sampling time and other sampling parameters are more important than total equilibration (Bulletin 923. Supleco website www.sigmaaldrich.com/Graphics/Supelco/objects/4600/4547.pdf).

However, ensuring that extraction times are as accurate as the manual sampling procedure will allow still remains prudent. Choosing the 20 minute exposure time also ensured that losses via possible evaporation or microbial degradation were kept to a minimum (these types of losses can cause problems with longer exposure times, Pawliszyn, 1999).

NB consideration must be given to the response of the different fibre types to the same chemicals. For example, Table 14 shows the *normalised* peak areas for the red and blue fibres – differing percentages are seen for each of the compounds. So, although both fibres pick up all the peaks, they do so in slightly different proportions.

Extraction temperature / Sample: Headspace ratio / Desorption temperature

This part of the investigation highlights the critical importance of method optimisation when using SPME. The high variation seen at the outset, both when the actual spraint sample and the hydrocarbon standard were sampled using the red SPME fibre contrasted starkly with the much lower variation produced by direct injection. Following adjustment of the extraction / desorption method, a much better result was seen and a similar range of variation was then seen when the blue SPME fibre was used with the adjusted method.

Adjustments to the SPME extraction / desorption method included:

Sample: headspace ratio - this changed from 1:3 to 1:1.Advice from a Supelco seminar session recommended vial size and sample volume to remain constant in order to ensure precision with SPME; sample; headspace ratio of 1:1 was also suggested. Yang & Peppard (1994) found that increasing the sample volume, while keeping the ratio of sample to headspace constant (1:1), increased analyte adsorption by either immersion or headspace SPME.

Temperature - extraction in the lab at room temperature was altered to extraction in an incubator at a constant 23°C. SPME analysis on a lab bench will be at ambient temperatures which may vary. This source of variation in the method is easily remedied by carrying out the extraction under controlled temperature conditions in an incubator. 23°C was chosen as this was approximately equivalent to the *maximum* temperature a spraint would experience in Britain (the mean highest temperature in the UK from 1971-2000 occurred in the month of July: 21.8-23.2°C. http://www.metoffice.gov.uk/climate/uk/averages/19712000/tmax/7.gif). It was thought that temperatures higher than this could affect the nature of the spraint material and the result obtained. Also, increases in extraction temperature causes an increase in the extraction rate, but simultaneously a decrease in the distribution constant (Pawliszyn, 1999).

Injector temperature – this changed from 250°C to 270°C. Recommended desorption temperatures for the two SPME fibres are 250°C for the red fibres and 260°C for the blue fibres. This was increased slightly to increase the acceleration of volatile analyte desorption from the fibre coating.

Desorption time – this increased from 2 minutes to 5 minutes. This was increased slightly to ensure that all volatile adsorbed to the fibre were released.

Introduction of inlet liner - standard gas chromatographic injectors usually have large volume inserts suited to a normal liquid injection and the vapours of the solvent used. Large volume inserts are not required with SPME as no solvent is introduced during desorption. Rapid analyte transfer from the injector to the column requires high carrier gas linear flow rates around the fibre coating. Reducing the internal diameter of the injector insert, so that it closely matches the outside diameter of the coated fibre, ensures that this happens (Pawliszyn, 1999).

Even with the improved results due to method improvement it was observed that *absolute* values of analytes still varied to an extent, however, looking at the tables of normalised values it can be seen that the relative proportions of analytes show much less variation.

CONCLUSIONS

- The red (100µm polydimethylsiloxane) and blue (65µm polydimethylsiloxane /divinylbenzene) SPME fibres were found to be most effective for use in spraint headspace analysis.
- Direct injection of chemicals delivers higher concentrations than headspace Solid Phase Micro Extraction (SPME). Differing volatilities mean that headspace concentrations will not necessarily match those found in the body of the liquid mixture.
- Increasing fibre exposure time is initially correlated with an increase in the amount of chemicals adsorbing to the fibre. After an exposure of 20 minutes the amount of chemicals adsorbing to the fibre levels off; possibly indicating a state of equilibrium.
- A sample to headspace ratio of 1:1 helped to produce more precision in the SPME method.
- Steady temperatures during extraction also helped to produce more precision in the SPME method.
- Increasing the GC injector temperature, increasing the fibre desorption time and introducing an SPME inlet liner all contributed to a more precise result.

CHAPTER 3

SPRAINT AGEING

INTRODUCTION

Deposited scent marks are designed to last a long time in the absence of the sender. Knowledge of the age of a spraint, would provide a temporal history of an individual and this type of information would be beneficial to both the receiver ('is the scent mark still relevant to the territory?') and the sender ('alerts the receiver to recent territorial scent marks') (Buesching et al, 2002b). Scent mark age would be of particular interest during the breeding season, allowing the location of recently occupied areas (Buesching et al, 2002b).

Emission rate and longevity of an olfactory signal can be substantially influenced by: (i) the chemical profile of the scent mark; (ii) the type of substrate; and (iii) the environmental conditions (Bradbury & Vehrencamp, 1998) (For further information see Chapter 1).

The chemical composition of sub-caudal gland secretions produced by a mustelid cousin of the otter, the European badger (*Meles meles*) has been studied (Buesching et al, 2002b) in relation to changes with length of time exposed to the environment. Sub-caudal gland secretions are mainly comprised of slow releasing long-chain, non-volatile carboxylic acids (Gorman et al, 1984). This type of secretion will therefore persist in the environment and as such would be useful as long term remote signals (Buesching et al, 2002b). The results showed that chemical composition of the sub-caudal secretion remains relatively stable over time when exposed to the environment. However, two components of the secretion were negatively correlated with time providing a possible mechanism for determining the scent mark age. For 'cheat-proof' territorial signals, comparison of the rate of decay of one volatile component with another that decays at a different rate is required. The ratio between the two would be continuously changing with time, and would be independent of the deposited scent mark size (Beynon & Hurst, 2003).

Few investigations of the effect of ageing on the chemical profile of European otter spraints have been undertaken. A single incident during studies of otters in Sweden saw a territory reoccupied by a new resident male; just 7 days after the previous resident had been shot (Erlinge, 1967b, 1968). Changes in spraint composition over time have also been studied; esters were shown to decay whilst sterols and fatty acids were more stable providing a possible mechanism for distinguishing spraint age (Fasano & Milone, 1993).

Rozhnov & Rogoschik (1994) investigated the effectiveness of spraint odour over time. The ability of the test otters to distinguish between their own fresh spraints and other fresh spraints was used as the assessment criteria. The study failed to demonstrate the otters' ability to distinguish between fresh and old spraints as there was a similar reaction to both. However it was shown that otters can distinguish between their own old spraints and other old spraints even when 60 days old. In the field, spraints have been shown to persist in the environment for up to 8 weeks (Mason & Macdonald, 1986), i.e., similar to the longevity of the olfactory message - at least 60 days (Rozhnov & Rogoschik, 1994).

Spraint longevity and therefore the longevity of the inherent olfactory information depends on a variety of factors already discussed above (chemical profile / substrate type / environmental conditions). Otters are known to deposit spraints under bridges (Kruuk, 1992) which would increase the longevity of the olfactory message. Spraints are also more likely to be found in wooded areas (Jenkins & Burrows, 1980) and can last for up to a year when deposited in sheltered areas (Jenkins & Burrows, 1980; Mason & Macdonald, 1986).

This study aims to map (using both solvent extraction and Solid Phase Micro-Extraction of the odour chemicals) how the chemical composition of otter spraint material changes in the days following deposition, how storage at low temperatures affects these changes and how the spraint material is affected when exposed to the environment.

MATERIALS AND METHODS

The animals

European otter samples were collected from a total 6 otters housed the *Bowes otter sanctuary, North Pennine Reserve, Bowes, County Durham*, run by The Otter Trust. The Otter Trust is the largest otter conservation body in the world and has been responsible for re-introductions of captive-bred otters into the Eastern half of Britain since 1983.

See Table 1 for more detailed information regarding the test animals and their diet

Table 1. Bowes sanctuary European otter information

Pens	A	B	C
Dimensions (*yards*)	20x18	20x18	18x28
Otter	Flame	Wishful, Torridge & cub.	Tilly & cub
Sex	male	female, male & male	female & female
DOB	24/06/94	24/04/94, 12/06/94	23/10/91
History	captive bred	captive bred	captive bred
Breeding History	1 cub		
Diet			
Feeding times	Food		
09:00 – 09:30	2, dead day old chicks		
12:00 + 15:30	Filleted fish (variety of spp.) – 'token titbits' 7 – 8 ounces for public feeds		
17:00 – 17:30	ZF6 (zoo food – see footnote) – adults eat ½ tin + scraps (fish heads plus more chicks). Extra eaten in winter – up to 3lb.		

- Canned ZF6 - food for wild animals 400g (based on kitten food with rabbit, chicken and herbs) - a complimentary, basic food for carnivorous wild animals.
- All pens contained a deep pool of freshwater for swimming.

Sample collection

At the sanctuaries those spraints/scats that appeared most fresh (confirmed by the keeper as deposited that day) were collected. Whole spraints were taken, kept intact wherever possible and placed in airtight, labelled glass containers. These were then placed over ice in a large insulated container and transported back to the lab the same day. Once at the lab the samples were immediately frozen to -70ºC in order to minimise any deterioration prior to analysis.

See Table 2 for details of sample collection.

Table 2. Sample collection details

Sample position	Generally found near freshwater pools and lying-up shelters.		
Sampling date	23/08/2000		
Pen	A	B	C
Number of samples collected	1	4	2

This batch of captive otter spraints was used to investigate ageing of the spraint odour profile over time with both a solvent extraction technique (diethyl ether) and with Solid Phase Micro Extraction (SPME).

The single spraint sample from pen A was quite small and was not used in this study, leaving 6 spraint samples available. Each sample was subjected to different temperature / environmental treatments in order to investigate their effect on the ageing process (see Table 3):

Table 3. Temperature/Environmental Treatments.

Pen no / spraint sample	*Treatment no.*	*Treatment*	*Location*	*Air exposure*
C / ii	1	-70ºC	in freezer	airtight container
C / i	2	-20ºC	in freezer	airtight container
B / i	3	14ºC	in incubator	exposed to air
B / ii	4	20ºC	in lab	exposed to air
B / iii	5	outdoors	in shade	exposed to air
B / iv	6	outdoors	in open	exposed to air

Each temperature / environmental treatment was chosen for a specific reason. The -70°C and -20°C treatments were chosen in order to investigate the effect of storage

of the samples. The 14°C and 20°C treatments were chosen in order to investigate stability of spraint samples at slightly differing temperatures under relatively controlled conditions. Finally, the 'open / shade' and 'open / exposed' treatments were chosen in an attempt to mimic more closely natural conditions (otters are known to spraint in the open along river banks on prominent rocks, but also in more shady and sheltered positions, e.g., beneath trees or under bridges).

Sub-sample preparation

i. Spraint samples were removed from storage at -70ºC and defrosted to room temperature.
ii. Each spraint was weighed and labelled.
iii. Each was then split into 7 equal parts along its length by eye (1 for use in SPME, the remaining 6 for solvent extraction).
iv. One sub-sample from each spraint was taken for SPME, weighed and placed in a 4ml amber, screw top vial (with hole-cap and PTFE/silicone septa) and another was taken for the initial solvent extraction.
v. The remaining 6 were subjected to the different temperature treatments (those placed in freezers were placed in airtight glass containers and all others were placed in petri dishes minus their lids. (NB the spraints placed outdoors had petri dishes drilled with holes to allow drainage of rain and/or condensation).

Solvent extraction procedure

The solvent extraction procedure taken from Trowbridge (1983) and modified for extraction of spraint *sub*-samples is seen below:

i. 6 labelled glass jars with airtight lids were prepared containing 15ml distilled water.
ii. A single sub-sample from each spraint was weighed and the weight recorded. One sub-sample was then placed in each jar, which was agitated vigorously until a saturated aqueous suspension was obtained.
iii. The spraint suspensions were then transferred to labelled centrifuge tubes (tubes were first washed with distilled water) and centrifuged at 2000 rpm for 5mins.

iv. 10ml of resultant supernatant transferred to small separating funnels and 20mL di-ethyl ether added. Each funnel vigorously agitated for 1 minute.

v. Aqueous phase then decanted into original jars.

vi. Organic phase (di-ethyl ether) decanted into quick-fit round bottomed flasks (through filter paper - to prevent contamination from aqueous phase). Flasks stoppered to prevent loss of volatiles.

vii. Aqueous phase now re-added to the funnels and steps 4 to 6 repeated x3.

viii. $CaCl_2$ was added to each flask in order to remove any aqueous phase that may have been added inadvertently.

ix. $CaCl_2$ removed by decanting ether to another flask leaving $CaCl_2$ behind.

x. The collected organic phase was now evaporated to dryness using a rotary evaporator under vacuum at 30ºC.

xi. Approximately 2ml ether added to dry flask to re-dissolve extracted chemicals. This produced an amber liquid with an 'ottery' smell.

xii. Extractions transferred to 4ml amber, screw top vial (with hole-cap and PTFE/silicone septa) ready for GC injection.

xiii. SPME procedure

For each treatment and for each SPME extraction a blue fibre (65µm polydimethylsiloxane/divinylbenzene - PDMS/DVB) was exposed to the headspace of the designated vial sub-sample for exactly 20 minutes in an upright position at 23°C. Immediately after the extraction the vial sub-samples were replaced in their different locations.

The fibre was ‘conditioned’ (cleaned) between each analysis (260°C for 0.5 hours). A blank run was carried out following each conditioning procedure, before exposure to the spraint sample to ensure the fibre was clean.

Odour profiles were extracted using for the di-ethyl ether extractions for each temperature treatment at 0 / 2 / 4 / 8 / 16 days and SPME for each temperature treatment at 0 / 2 / 4 / 8 / 16 / 32 days. Due to the nature of the SPME technique (ability to use a single sub-sample over and over), an additional extraction was

decided upon. This was not possible with the solvent extractions as each sub-sample was completely utilised during the extraction process.

Instrumentation, technical specifications and equipment

Gas chromatograph

Hewlett Packard 5890 Series II - Helium carrier @ 2 mL/min (constant flow)

GC column

Column type, SGE, (Analytical Science) - Bonded Phase (BP1) non-polar; column dimensions, 25m x 0.33mm; temperature limits; -60 to 340°C; material, fused silica; film thickness, 0.25µm; I.D., 0.22mm.

Temperature programme

The chromatograph was run in a splitless mode and using the following additional parameters: injector temperature 270ºC; detection temperature 300ºC. Initial oven temperature 35ºC for 2 min; this was then increased by 5ºC/min up to 170ºC, this temperature was maintained for 2 min; then there was a further increase of 15ºC/min up to 270ºC, again maintained for 2 min; fibre desorption time was 5 min.

Only peaks from the first 30 minutes of each chromatogram were investigated (peaks appearing later than this were thought to be more likely to be products of column or fibre bleed due to the higher temperatures reached). The 'area' of each peak is directly proportional to the *concentration* of that chemical in the sample extract. Untransformed peak area data was examined and these were also normalised providing a measure of the relative concentration of each chemical. This allowed the fluctuations in the profile over time to be assessed and compared to the changing profiles seen under different temperature treatments.

Controls

SPME

The SPME fibre was exposed to laboratory air and run through the GC in the same manner as the samples in the ageing experiment. The peaks caused by the compounds in the laboratory air could therefore be subtracted from the chromatograms of the test samples.

The SPME fibre was also exposed to an empty vial (washed in distilled water and dried in drying oven) and then run through the GC. Again, any peaks caused by the compounds from the PTFE/Silicone Septa in the cap of the sample vials could be subtracted from the chromatograms of the test samples.

Solvent extraction

In the same way that laboratory air compounds will contaminate the samples when using SPME, distilled water compounds will contaminate the samples when carrying out the di-ethyl ether extractions. Therefore, a control was required here also. The extraction procedure was followed using only distilled water and no spraint sub-sample, this was again analysed with GC in the same manner as the samples in the ageing experiment. The peaks caused by the compounds in the distilled water could therefore be subtracted from the chromatograms of the test samples.

Outside temperatures

Temperature measurements were taken on each sampling day (0, 2, 4, 8, 16, 32 days), one at midday and one at midnight. This was carried out in both the open position the shaded position. Mean temperatures were calculated for both positions.

Spraint weight loss over time

Weights (g) of the spraint samples were taken during the ageing experiment at 0 / 2 / 4 / 8 / 16 / 32 days. These were plotted against time.

RESULTS

Di-ethyl ether extractions

Untransformed data

The data for each treatment (-70°C; -20°C; 14ºC; 20ºC; open/shade; open/exposed) all showed similar fluctuations in peak area for each chemical.

Despite not all peak data (only the first 30 minutes of each chromatogram) being used, it is evident that each spraint odour profile is extremely complex. This made it very difficult to observe any patterns present in the data using graphical means, compounded by the fact that each graph contained a varying number of peaks.

Many of these peaks showed an upward trend across all treatments. The largest peak (**P23.57**) also showed an upward trend and one peak (**P25.59**) appeared to be more stable. These two peaks were singled out and examined in more detail (see Figures 1-6). Lines of best fit were added to each data set in order to observe the general trend over time.

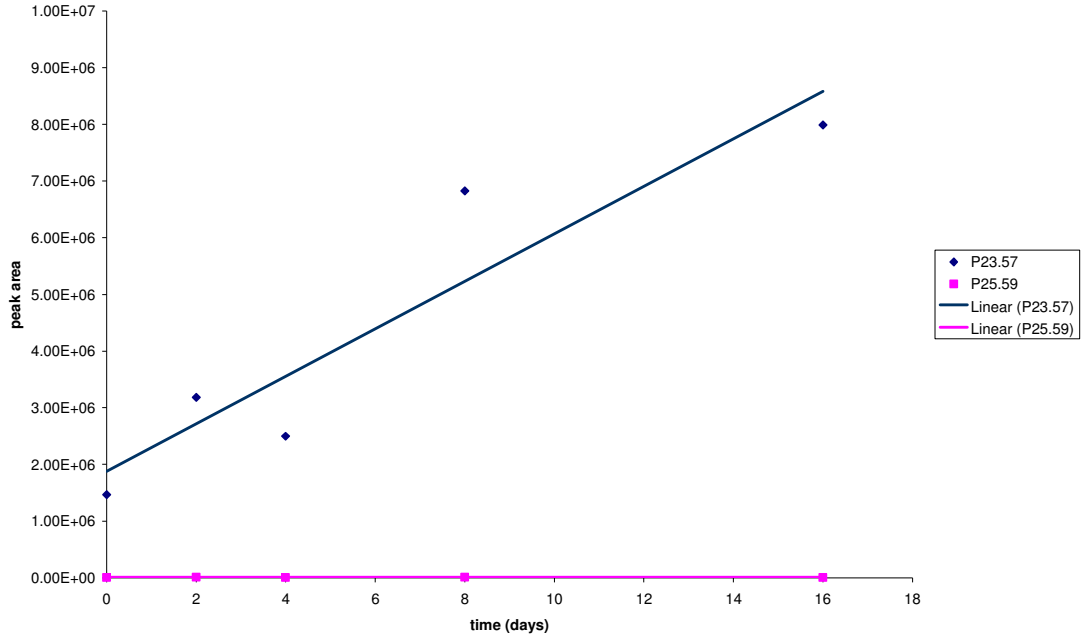


Figure 1. Di-ethyl ether extractions – ageing of untransformed data for P23.57 and P25.59 at -70 ºC

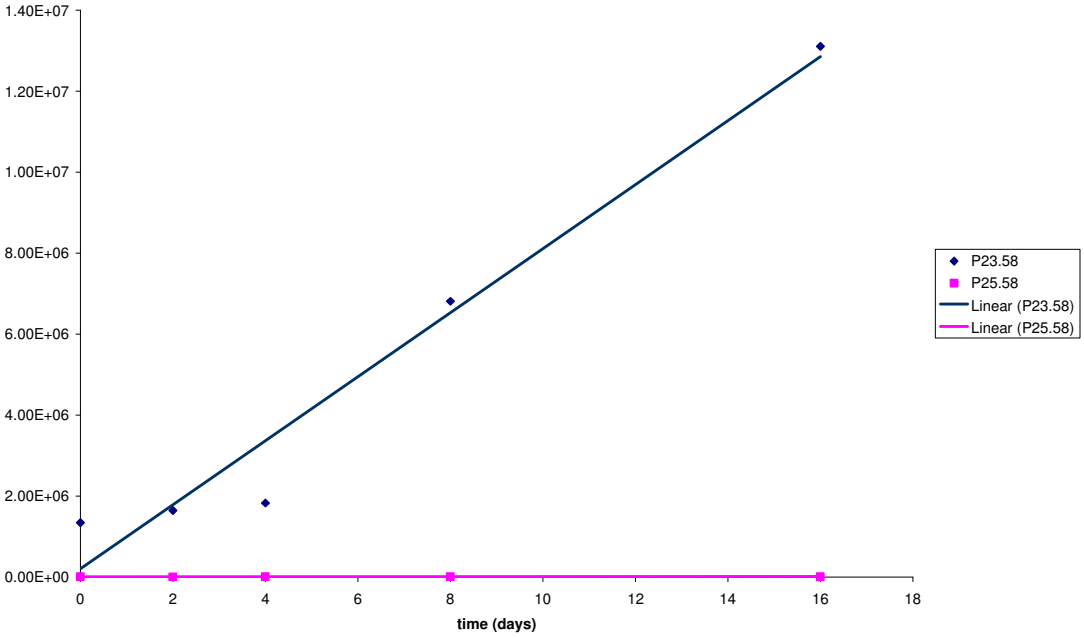


Figure 2. Di-ethyl ether extractions – ageing of untransformed data for P23.57 and P25.59 at -20 ºC

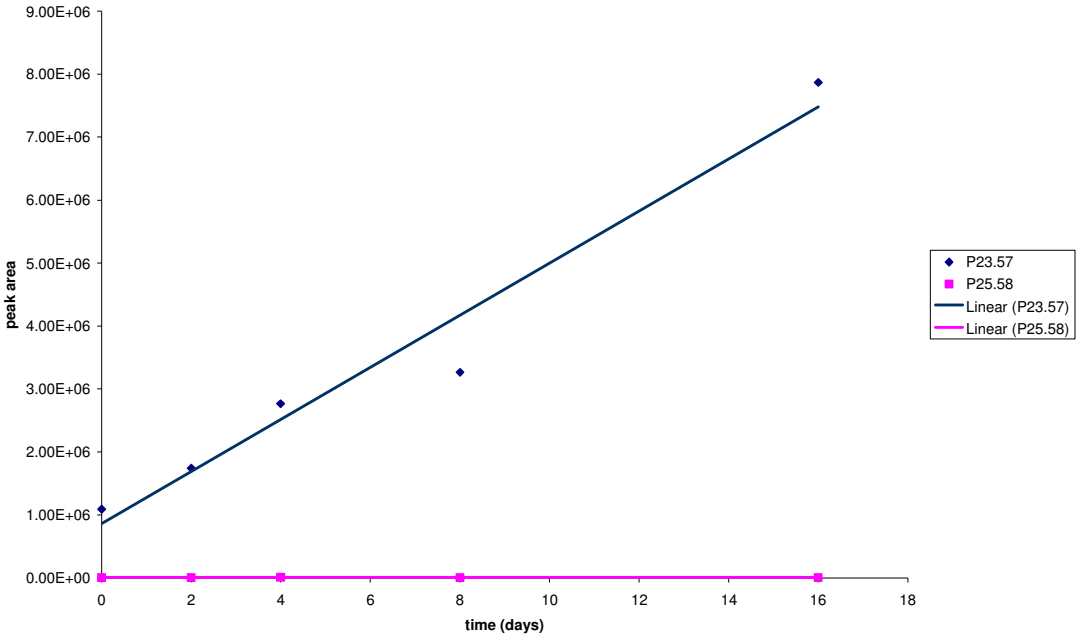


Figure 3. Di-ethyl ether extractions – ageing of untransformed data for P23.57 and P25.59 at 14 ºC

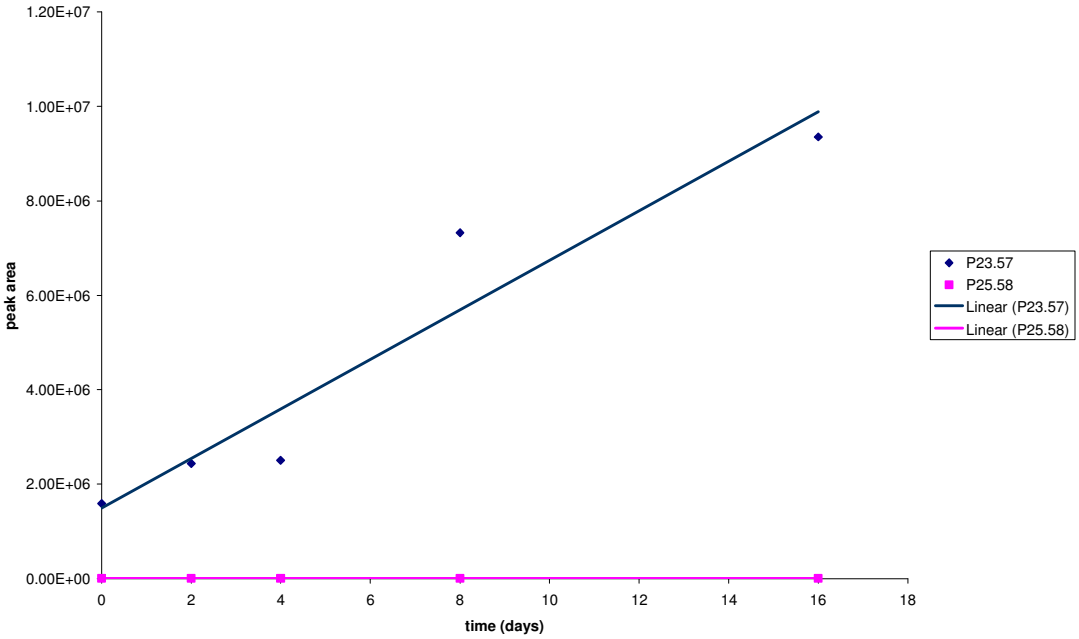


Figure 4. Di-ethyl ether extractions – ageing of untransformed data for P23.57 and P25.59 at 20 ºC

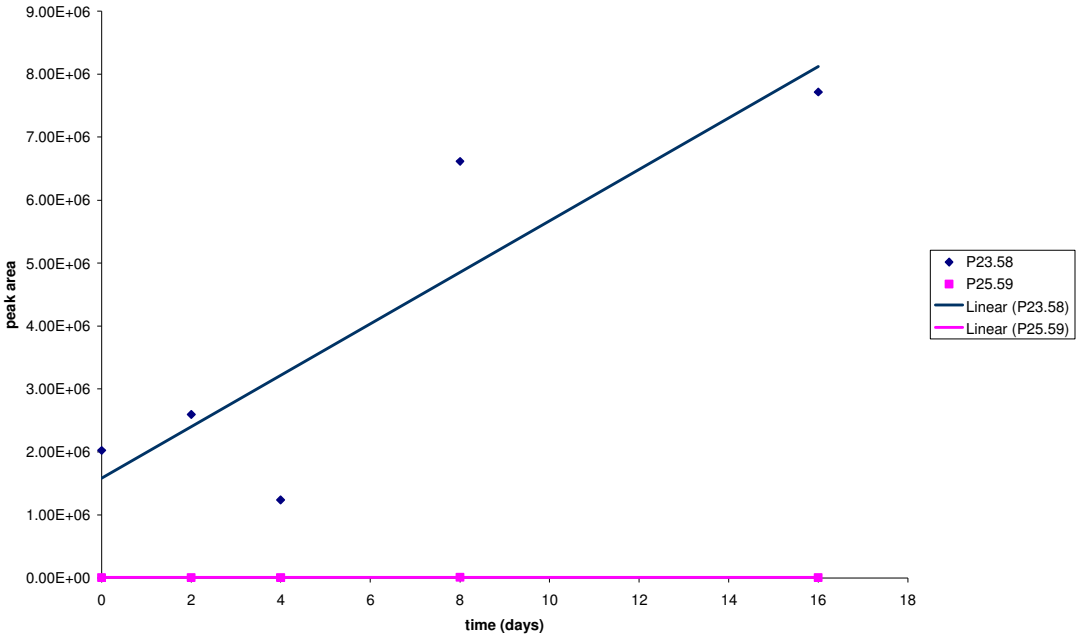


Figure 5. Di-ethyl ether extractions – ageing of untransformed data for P23.57 and P25.59 in open / shade

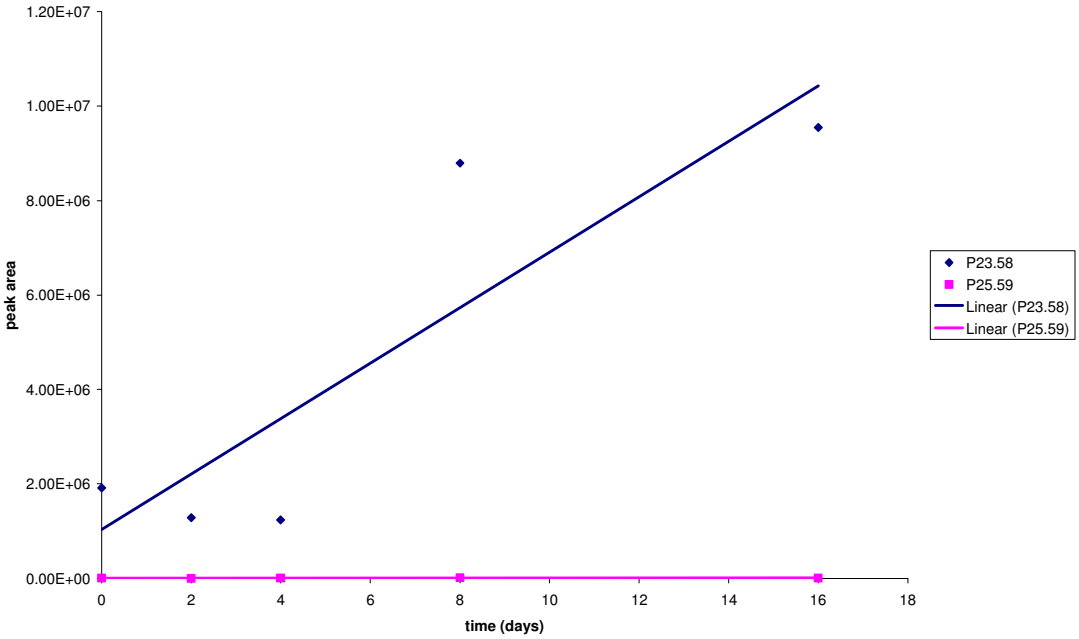


Figure 6. Di-ethyl ether extractions – ageing of untransformed data for P23.57 and P25.59 in the open / exposed

For the -70°C treatment there was an upward trend in the peak area of P23.57 over time. In direct comparison the peak area of P25.59 appeared to remain fairly stable. A very similar result was seen in the remainder of the treatments with the gradient of the best fit line for the upward trend of P23.57 looking similar in each case.

Normalised data

Normalising the data altered the trends seen with the untransformed peak areas. **P23.57** and **P25.59** look fairly stable with a very slight downward trend. These peaks were again examined in more detail (see Figures 7-12) as a comparison to the untransformed peak area data.

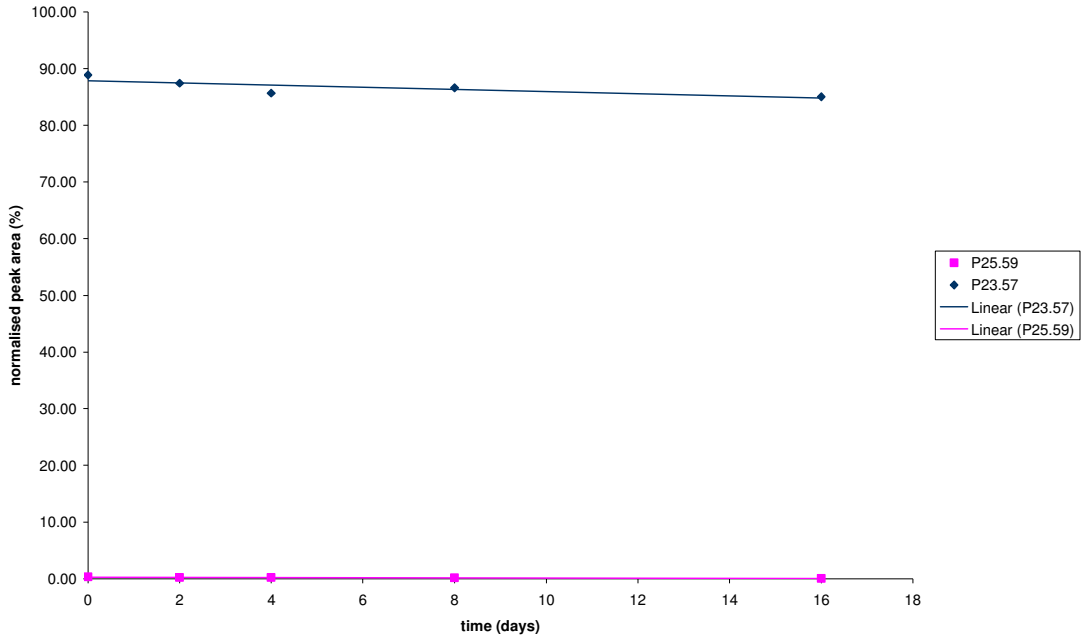


Figure 7. Di-ethyl ether extractions – ageing of normalised data for P23.57 and P25.59 at -70 ºC

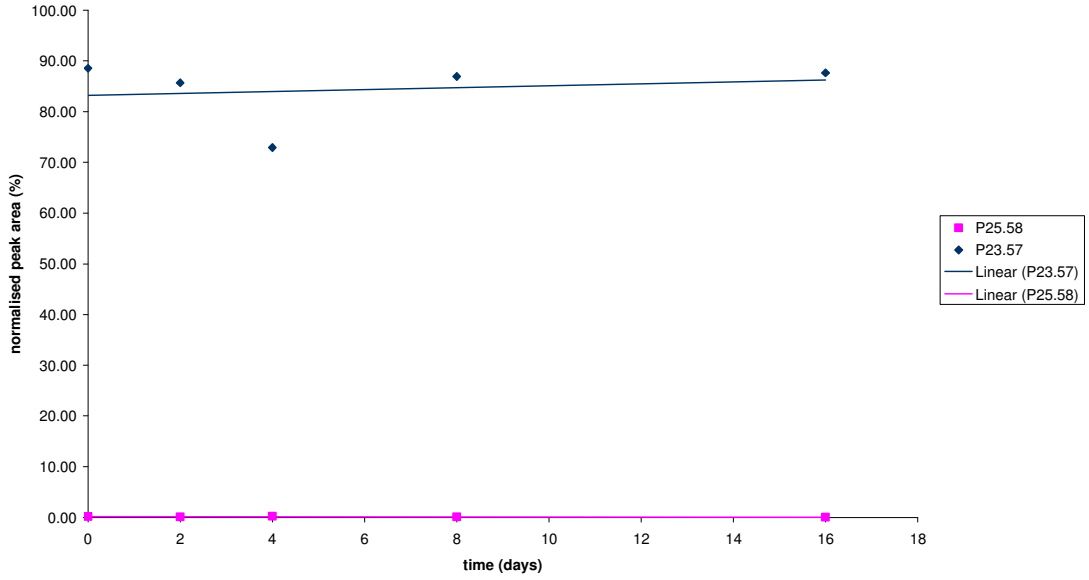


Figure 8. Di-ethyl ether extractions – ageing of normalised data for P23.57 and P25.59 at -20 ºC

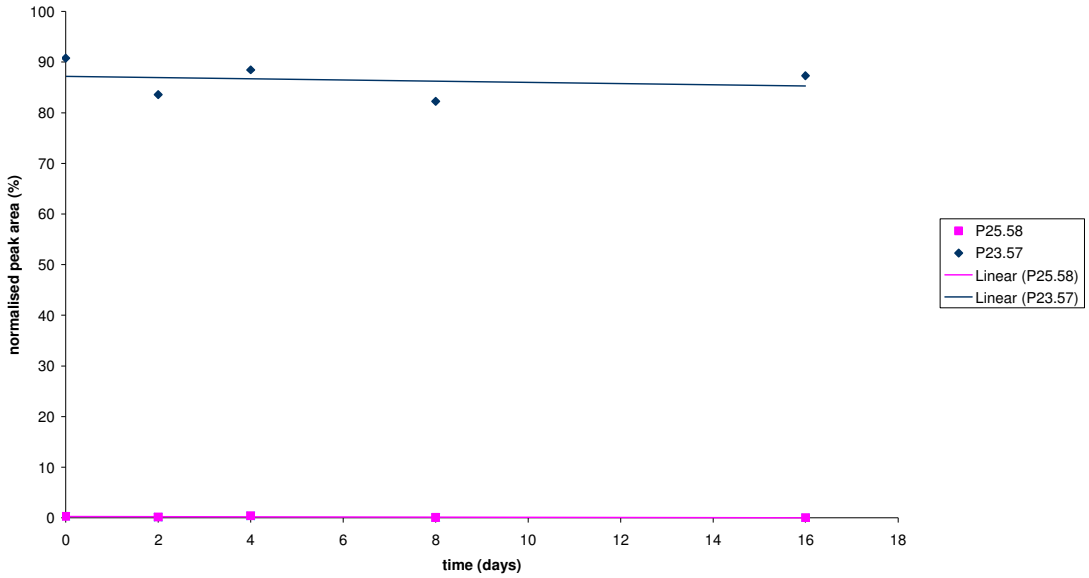


Figure 9. Di-ethyl ether extractions – ageing of normalised data for P23.57 and P25.59 at 14 ºC

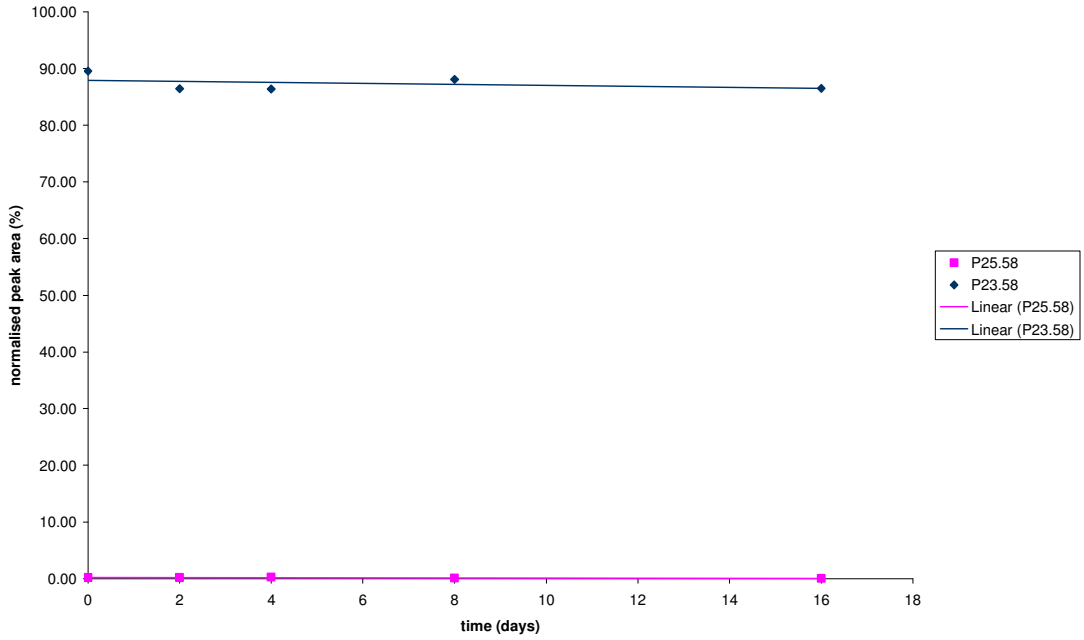


Figure 10. Di-ethyl ether extractions – ageing of normalised data for P23.57 and P25.59 at 20 ºC

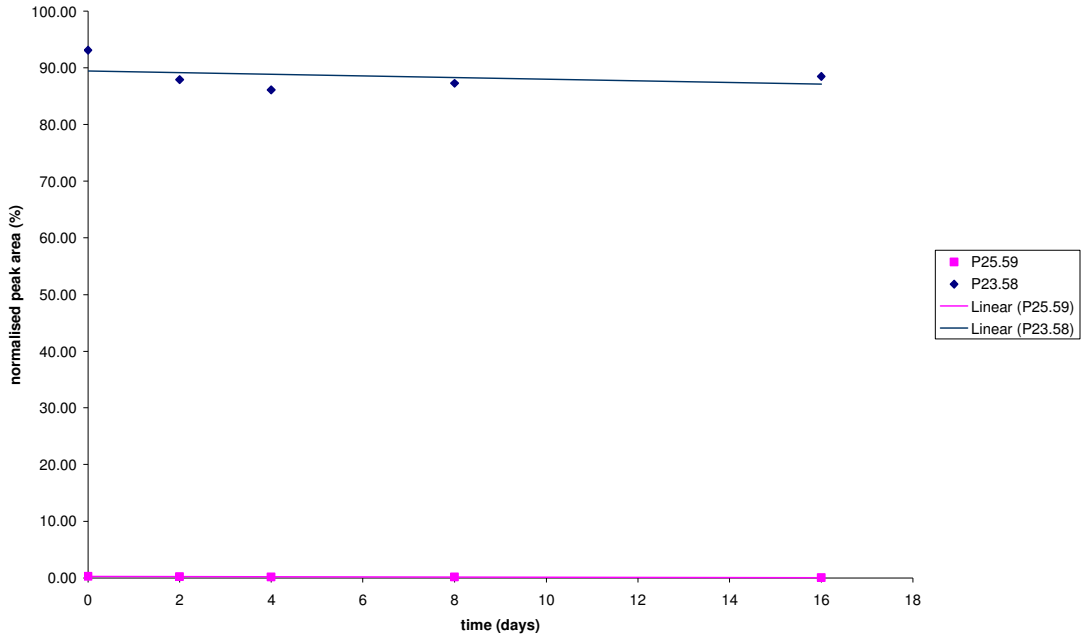


Figure 11. Di-ethyl ether extractions – ageing of normalised data for P23.57 and P25.59 in open / shade

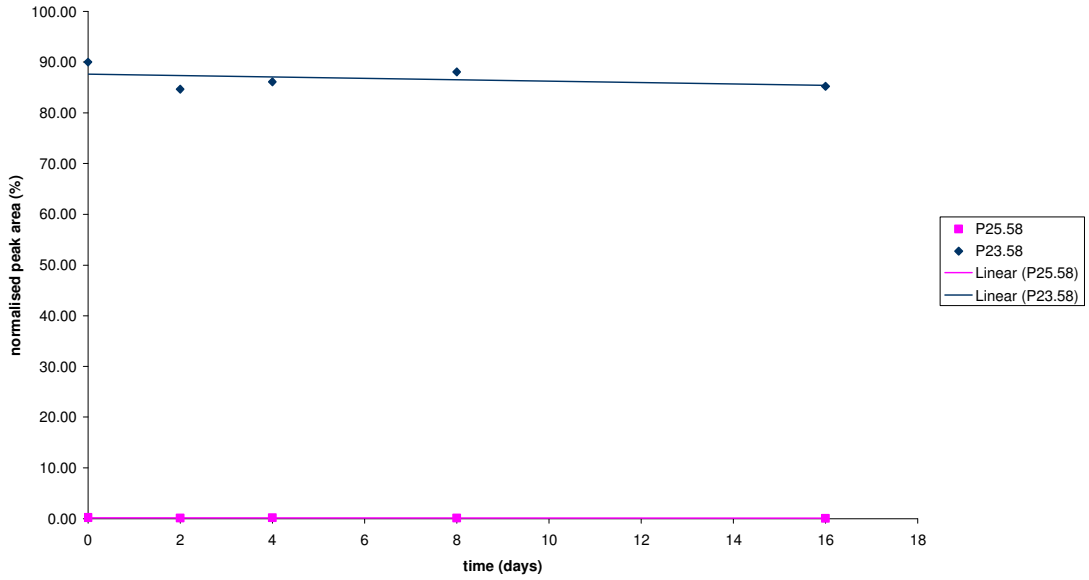


Figure 12. Di-ethyl ether extractions – ageing of normalised data for P23.57 and P25.59 in the open / exposed

For the-70°C treatment the normalised peak area of P23.57 appeared to remain fairly stable across the time period. The normalised peak area for P25.59 dropped away slightly towards day 16. This showed a similar pattern across all the treatments.

To sum up the solvent extraction section of the results: untransformed data for the major peak (P23.57) saw a similar upward trend across all treatments, however, in relative terms (normalised data) the proportion of P23.57 remained very similar throughout, dropping off only slightly. This was due to the contribution of other peaks that also showed an upward trend in the untransformed data together with that of peaks that only appeared after 4, 8 or 16 days. The untransformed peak area data for P25.59 (in direct comparison with P23.57) remained relatively stable across all treatments. The normalised peak area data seemed to be similarly stable in direct comparison with the data from P23.57; however these figures actually showed a downward trend over the time period.

SPME extractions

Untransformed data

The SPME data contrasted to the di-ethyl ether extraction data, which showed each treatment to have similar fluctuations in peak area for each chemical.

The spraint samples stored at -70ºC remained relatively stable over the study period. There was however, a certain level of fluctuation in the relative peak concentration of each chemical.

At -20ºC, whilst most of the peaks remained relatively stable one peak in particular (peak 13, retention time: 18:11) declined over the study period.

Both samples might also have undergone decay (possibly through loss of volatiles or through microbial activity) of the odour chemicals despite the low temperatures involved.

The graphs for all other treatments (14ºC; 20ºC; open/shade; open/exposed) showed relatively more fluctuation in relative peak proportion for each chemical than for the frozen sub-samples.

Again, it is clear that each spraint odour profile is extremely complex and this made observation of patterns present in the data using graphical means problematic.

Examining the data it became evident that 2 peaks (**P10.52** and **P18.11**) generally exhibited the largest fluctuations in peak area over the study period. These peaks were singled out and looked at in more detail (see Figures 13-18).

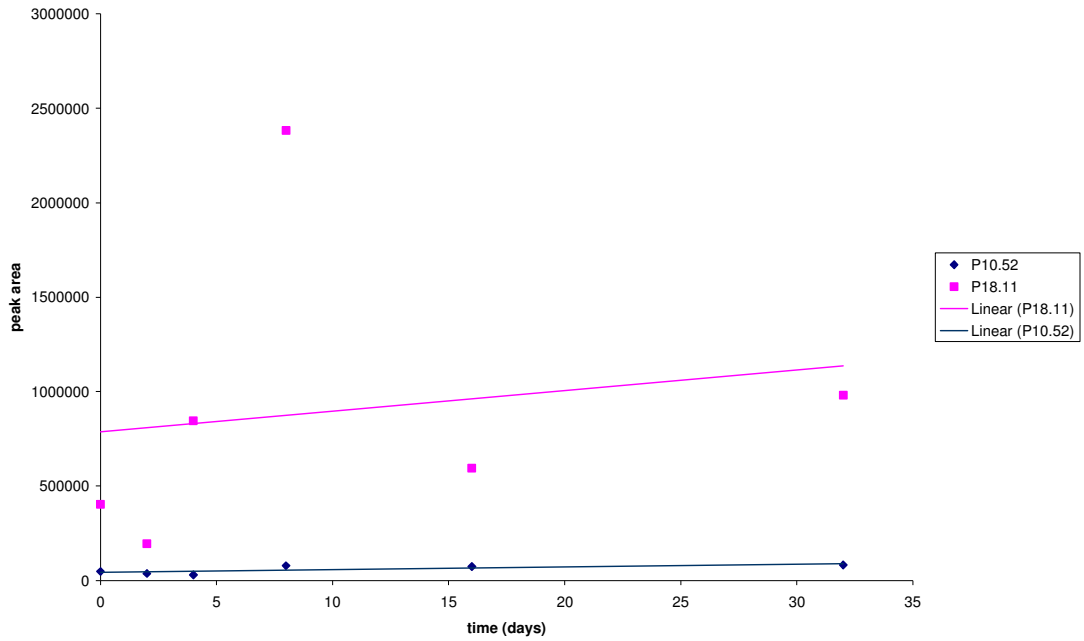


Figure 13. SPME extractions - ageing of untransformed data for P10.52 and P18.11 at -70 ºC

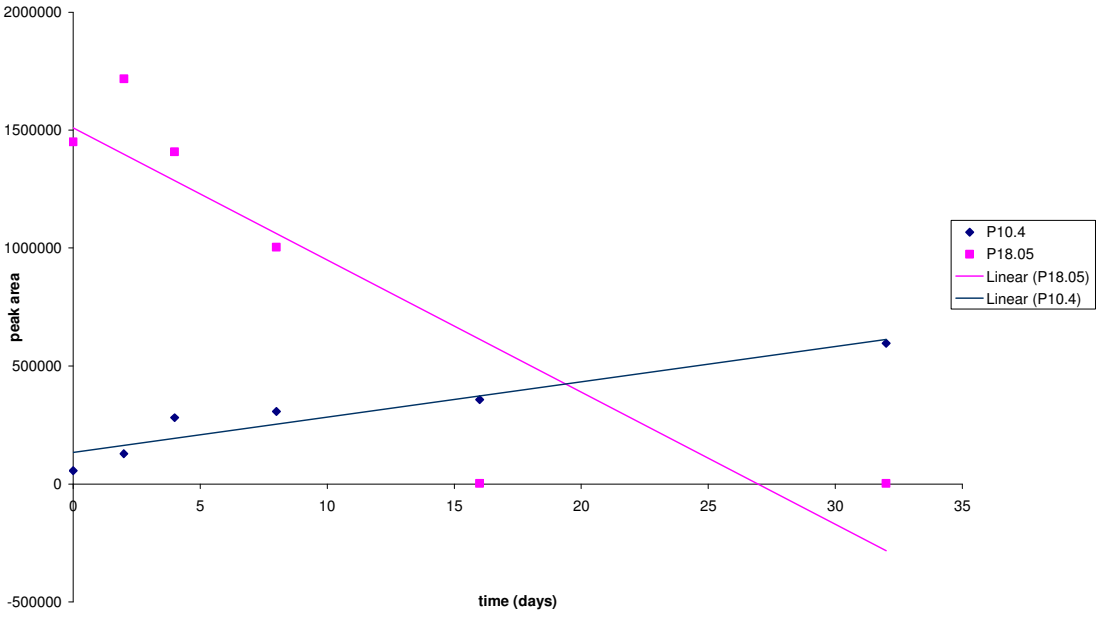


Figure 14. SPME extractions - ageing of untransformed data for P10.52 and P18.11 at -20 ºC

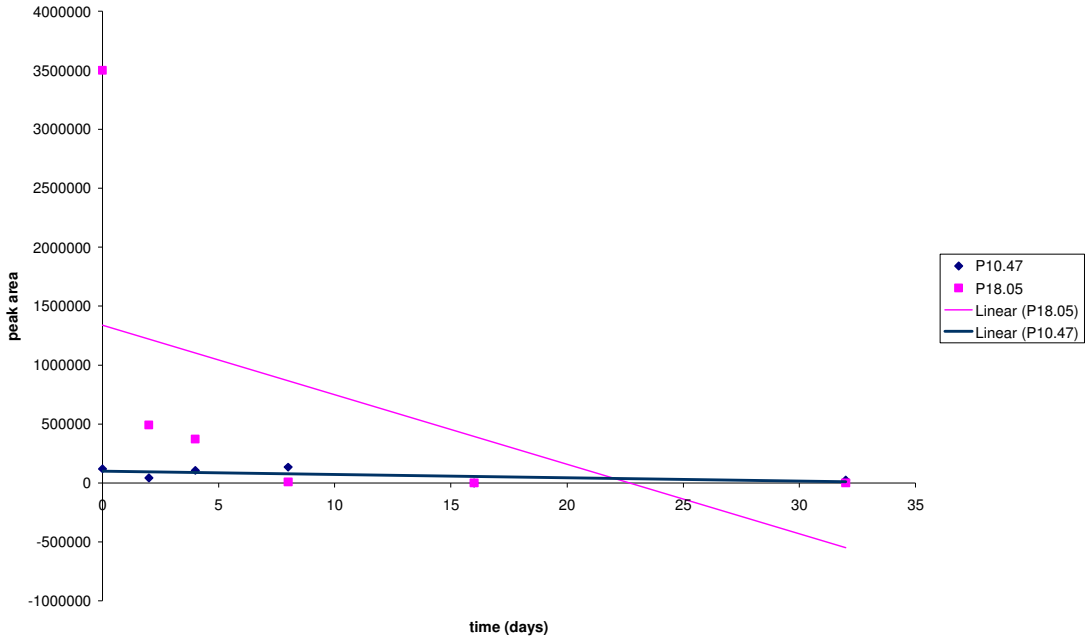


Figure 15. SPME extractions - ageing of untransformed data for P10.52 and P18.11 at 14 ºC

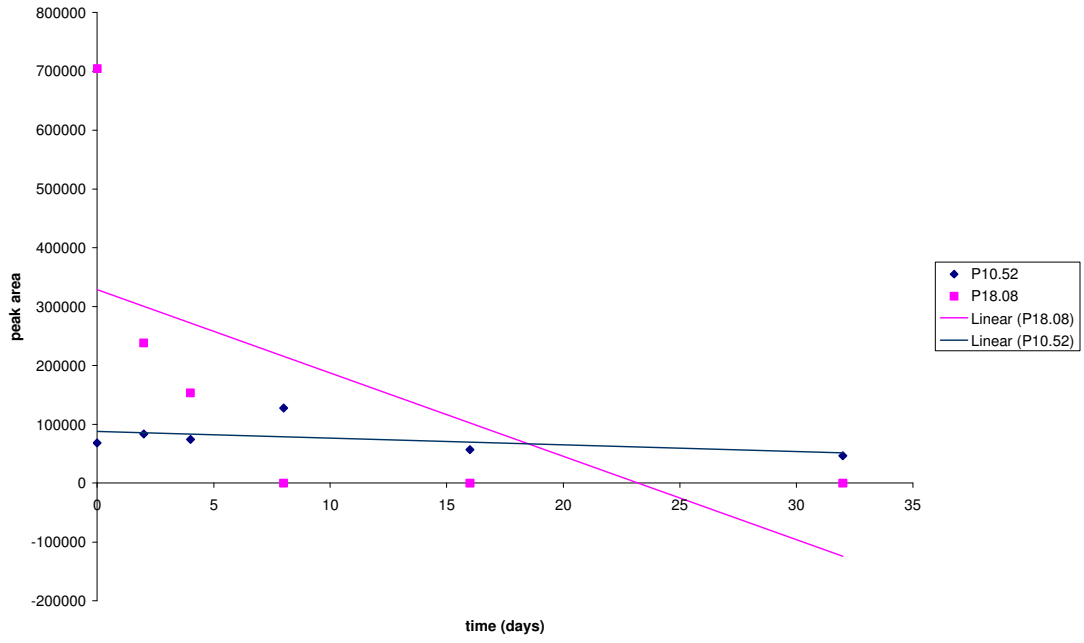


Figure 16. SPME extractions - ageing of untransformed data for P10.52 and P18.11 at 20 ºC

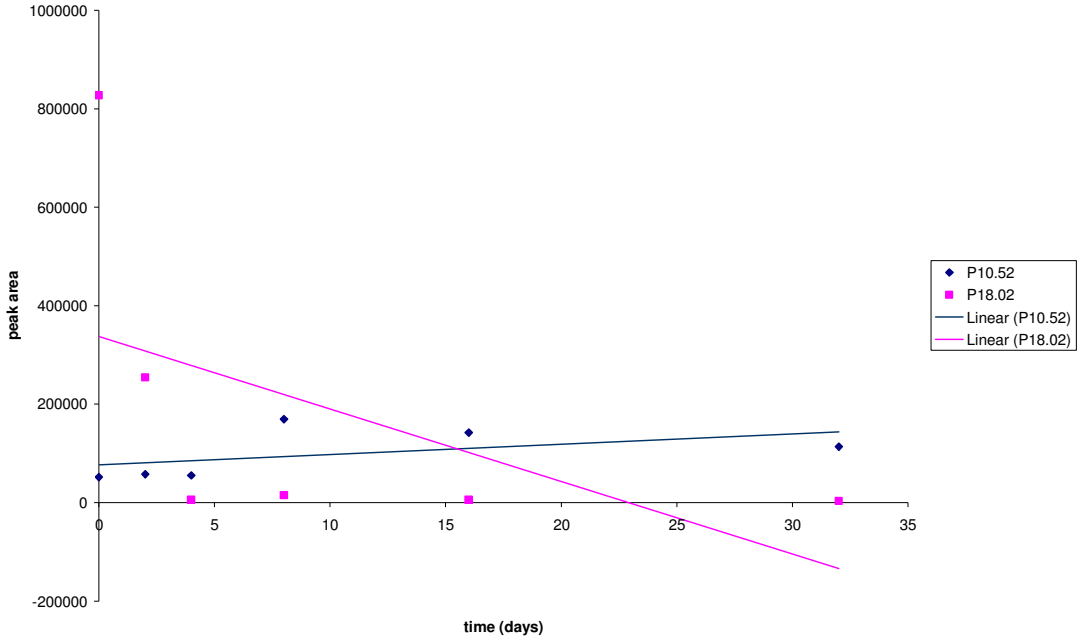


Figure 17. SPME extractions - ageing of untransformed data for P10.52 and P18.11 in open / shade

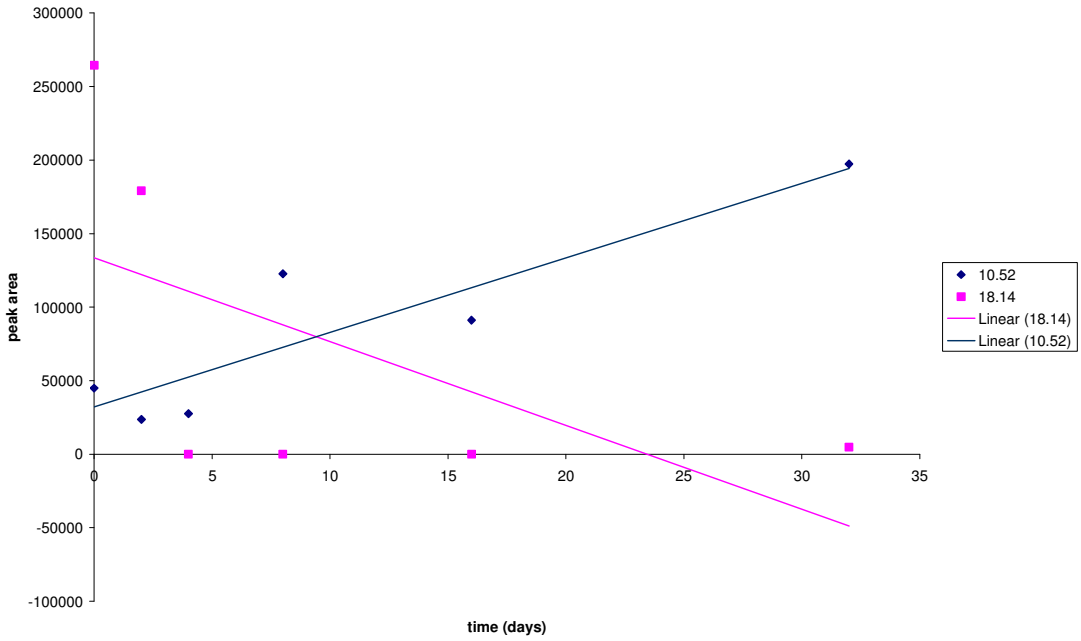


Figure 18. SPME extractions - ageing of untransformed data for P10.52 and P18.11 in open / exposed

Most treatments, apart from the -70, show similar trends for both these peaks: a general decay in the untransformed peak area over the study period for P18.11 and an increase in the untransformed peak area of P10.52.

Peak 18.11

Comparing the graphs, there was a similar initial rate of loss of untransformed peak area over the first 2-4 days of the study in all treatments apart from the -70ºC treatment. Subsequently, all treatments (again, apart from -70ºC) show the untransformed peak area levels eventually falling away to either to zero or to a very low level. The -70ºC treatment showed some fluctuation of P18.11 over the 32-day period, but remained relatively steady in comparison to the other treatments.

Peak 10.52

Comparing the graphs, most treatments (apart from -70ºC) showed an overall increase in untransformed peak area levels for P10.52 over the study period. The 20ºC treatment showed the biggest increase in untransformed peak area over the study period.

Due to the two peaks having opposite time dependence, the lines of best fit associated with their respective data sets crossed each other resulting in a 'crossover point' (CP). This was given a value in days (read from the x axis).

Throughout the study, as the data was recorded for each successive time period (i.e., day 2, day 4, day 8….etc), a similar graph was generated and lines of best fit added. This allowed an evaluation of how the CPs behaved at specific points in the ageing process. The CPs for all treatments and all time periods for the untransformed data can be seen in Table 4.

Table 4. Comparison of all crossover points for all treatments using untransformed data

	0 – 2 days	0 – 4 days	0 – 8 days	0 – 16 days	0 – 32 days
-70°C	-	-	-	-	-
-20°C	-	-	-	13.5	19
14°C	-	3.75	6.2	11.6	22
20°C	-	4	5.7	10.1	18.5
open / shade	-	3.7	5.3	9	15.2
open / exposed	-	3.9	4.8	7.4	9.5

There is no CP for the -70°C treatment. The CP for the -20°C treatment first appears after 16 days. For all other temperature treatments the CPs first appear after 4 days

The data for 0-4 days the CPs do not follow a gradient, but all fall within the following range of ~3.7 - 4 days.

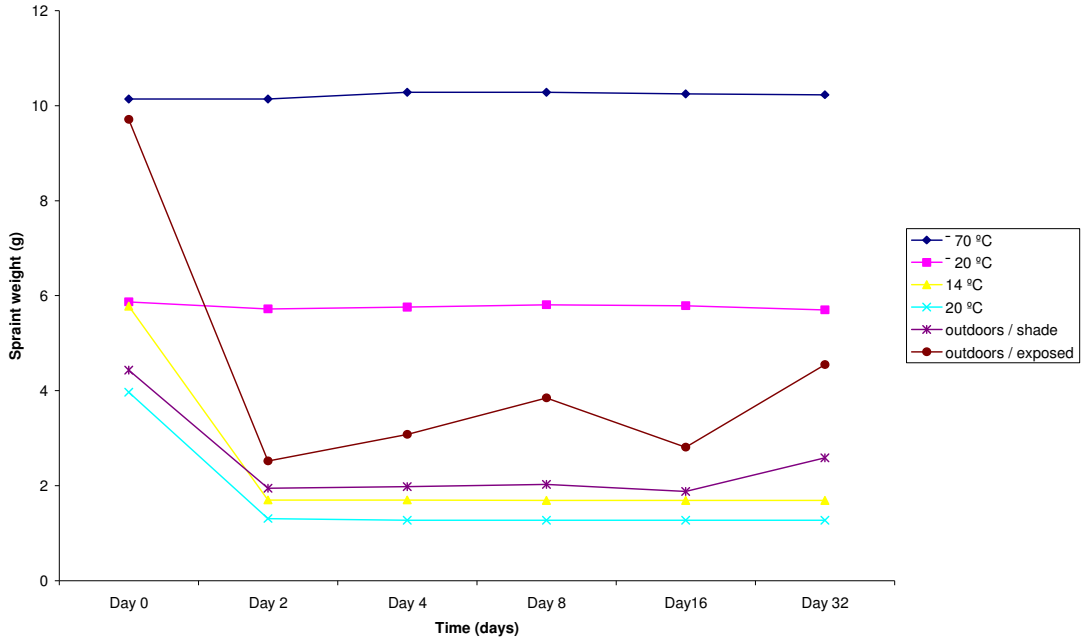
For 0-8 days, the open / exposed treatment has the greatest effect on the two peaks, producing a CP of ~4.8 days. This is followed by open / shade (~5.3 days), 20°C (5.7 days) and 14°C (6.2 days). The 0-16 day data shows the same pattern, except the CP for the -20°C appears also. The 0-32 day data follows the same pattern as the 0-16 day data, with one exception: the CP for -20°C (~19 days) is lower than the 14°C treatment (22 days).

Outside temperatures

The open / exposed position had a mean temperature of 16.2°C and the open / shaded position had a mean temperature of 12.6°C.

Spraint weight loss over time

Figure 19. Spraint weight loss (g) over time (days)



The spraint samples that were frozen (-70 ºC and -20 ºC) maintained a stable weight throughout the 32-day period. Samples in all other situations experienced substantial weight loss in the first 2 days (due to loss of water content, the appearance of the samples changed markedly from glistening and jelly-like to dry and dull). The initial rate of weight loss (between 0 and 2 days) differed between the treatments. The open / exposed treatment showed the fastest rate of water loss. Next came the 14°C treatment, followed by the 20°C treatment and the open / shade treatment, both sharing a similar rate of weight loss. From day 2 - 32 the spraint weight for the 2 indoor treatments (14°C and 20°C) remained steady and constant. Fluctuations were only seen in the weight of the 2 outdoor treatments (open / shade; open / exposed) due to rainfall between day 2 and day 4; day 4 and day 8; day 16 and day 32. The open / exposed spraint was affected much more than the spraint placed under cover.

All samples, despite the dramatic loss of hydration still retained their characteristic ottery smell. In addition, the odour of the outdoor spraint samples gradually faded during the study period to reveal a more fish-like aroma (personal observation). This occurred between the 8 and 16-day mark.

DISCUSSION

The di-ethyl ether extraction results contrasted with those for the SPME extractions in a number of ways. Most significantly, the main common peaks for each extraction procedure were different chemicals, having different retention times (see Tables 4.and 8.). This seems logical as many chemicals found in the body of the spraint will not necessarily be volatile enough to be found amongst the headspace chemicals, or may only be present there in very small amounts or even are hidden within the spraint as they have no direct contact with the air. For instance, if the anal sac secretions initially mask the odour of the undigested dietary material then food odour chemicals would not be found in the headspace and would therefore not be extracted with SPME. Oily sebaceous secretions are long lived and release their volatiles at a relatively slow rate, whereas, the more watery apocrine secretions are much more volatile (Gorman & Trowbridge, 1989). The volatility of chemicals found in the anal sac secretions is dependent upon the matrix they are held within. The lumen of otter anal sacs is filled with the secretions from the sebaceous and apocrine glands that surround it. Histochemical analysis of the secretion shows it to consist of protein and acidic mucopolysaccharide from the apocrine glands punctuated by lipid droplets from the sebaceous glands (Gorman et al, 1978).

All this indicates that the individual procedures may fail to show a complete chemical picture within both the spraint and its odour profile and that a combination of extraction procedures may be necessary.

Solvent extractions

These results focussed on two of the common peaks (i.e., from substances found in all spraint samples – P23.57 and P25.59).

Untransformed data

In the untransformed data, P23.57 was the largest peak seen and an increase in peak area was seen over the 16 day period. P25.59 was much smaller and was more stable in comparison. The different temperature / environmental treatments seemed to have little effect on the rate of deterioration of the chemicals found in the extraction odour profile. Despite the low temperature (-20ºC), microbial activity is still possible (Rivkina et al, 2000) and although this would not happen at -70ºC, the repeated

defrosting, heating and re-freezing in order to sample may have had an effect. The lines of best fit plotted for P23.57 on each graph, despite crossing the y-axis at different points, all appeared to have a similar gradient. Again this may be due to chemicals found within the body of the spraint that may not be affected by the temperature / environmental treatments as much as those found in the headspace.

Normalised data

The trends seen in the normalised data for P23.57 and P25.59 were different to those seen in the untransformed data. The normalised peak area for P23.57 remained fairly stable across the time period and the normalised peak area for P25.59 dropped away slightly towards day 16. The resultant graphs show a stark contrast between the untransformed and normalised data for these two peaks (untransformed: Figures 1-6 / normalised: Figures 7-12). The apparent stability in the relative amount of P23.57 present in the spraint material over time was due to the contribution of other peaks that showed an upward trend in the untransformed data in conjunction with peaks that only appeared after 4, 8 or 16 days. It is possible that this peak may function in individual or species recognition, remaining in a scent mark as a stable presence amongst other chemicals that may be increasing or decreasing.

SPME extractions

These results also highlighted two major common peaks (with different retention times to those seen with di-ethyl ether extractions - P10.52 and P18.11). These had an opposite time dependence. This offers up a possible spraint ageing mechanism, however, the different temperature / environmental treatments seemed to have a pronounced effect on the rate of deterioration of the particular chemicals found in the spraint odour profile (in contrast to those extracted with di-ethyl ether). Lower rates of decay and accumulation were seen at the lower temperatures and vice versa. This was reflected in the crossover points (CPs) for the two peaks (see Tables 14 and 15).

There was no CP for the -70ºC treatment the peaks remaining relatively stable throughout the study period, presumably due to the low temperature involved. The -20ºC treatment produced a degree of deterioration, with the first CP seen on the 0-16 day graph (~13.5 days) although this much less than was seen, with other treatments. Despite the low temperature (-20ºC), microbial activity is still possible (Rivkina et al,

2000). This together with the repeated defrosting, heating and re-freezing resulted in less stability than observed at -70ºC, but more stability when compared with the other temperature treatments (where the CPs occurred earlier in the experiment). The 14ºC treatment produced more deterioration, with the first CP seen on the 0-4 day graph (~3.75 days). The 20ºC treatment produced the first CP on the 0-4 day graph (~4 days). The open / shade treatment produced the first CP on the 0-4 day graph (~3.7 days). The open / exposed treatment also produced the first CP on the 0-4 day graph (~3.9 days). This result is as one might expect with increasing temperatures producing more deterioration in the spraint odour profile. The open exposed position had a mean temperature of 16.2°C, i.e., in a similar range to that of the indoor positions (14°C and 20°C). However, the spraints placed in outdoor conditions are more likely to experience microbial attack than those placed indoors, this coupled with the effects of sun, wind and rain may be possible explanations for the higher rate of change.

These results offer up a possible spraint ageing mechanism. Crossover points for the unfrozen spraints after 4 days stood between 3.7 - 4.0 (a range of 0.3). After 8 days CPs stood between 4.8 - 6.2 (a range of 1.4). After 16 days CPs stood between 7.4 - 11.6 (a range of 4.2). Finally, after 32 days CPs stood between 9.5 - 22 in the (a range of 12.5). It appears that the older a spraint is, the less accurate an estimation of age may be if these peaks were used as a measure.

Whilst the SPME headspace analysis is more similar to the way an otter would investigate a spraint, both extraction processes could be useful in the surveying of otter populations.

This part of the experiment has demonstrated that temperature can have an effect on the rates of production and deterioration of chemicals within the spraint odour. Environmental effects therefore govern to a large extent the rate of change of the spraint odour profile and this will affect accuracy of any estimate of age from spraints in the field (location, temperature, local weather conditions will all have to be taken into consideration).

Otters possibly use a mechanism similar to the one suggested here, i.e., comparing levels of different compounds, in order to age spraint deposits. This is important because in territorial marking terms, a signal made up of a single volatile component is not ‘cheat-proof’, as a small recently deposited signal cannot be distinguished from

a larger signal deposited relatively less recently (Beynon & Hurst, 2003). This would have implications for the advertisement of territory ownership. For a cheat-proof signal, comparison of the rate of decay of one volatile component with another that decays at a different rate is required. The ratio between the two would be continuously changing with time, and would be independent of the deposited scent mark size (Beynon & Hurst, 2003). Careful placement of the spraint may confer an advantage to the signalling animal however. As the results showed, more deterioration is seen with higher temperatures and with increasing exposure to other environmental conditions (e.g., sun, wind, rain). Otters are likely to deposit spraints in wooded areas (Jenkins & Burrows, 1980) and also under bridges (Kruuk, 1992). This would prolong the life of a scent mark and allow less frequent replenishment (important in terms of energy conservation, both in terms of manufacture of secretion and of movement between sprainting sites).

If a mechanism such as this were to be used in order to map otter movements by human researchers, then the influence of environmental factors would have to be taken into consideration to gain as accurate an estimation of age as possible.

NB this result (albeit without chemical identification) seems to concur with another study into spraint decomposition over time (Fasano & Milone, 1993); esters diminished whilst sterols / fatty acids were more stable

Spraint weight loss over time

Samples allowed to age, experienced substantial weight loss in the first 2 days. Due to loss of water content, the appearance of the samples changed markedly from glistening and jelly-like to dry and dull. This type of oxidation effect (colour change seen over time exposed to the environment) has been observed in aardwolves, *Proteles cristatus* (Silwa, 1996), peccaries (Sowls, 1984) and brown hyaenas, *Hyaena brunnea* (Mills et al, 1980).

These results not only illustrate the dramatic loss of hydration the spraints can undergo in dry conditions, but also that a degree re-hydration is possible in wet conditions. Considering P18.11 the dramatic spraint weight loss in the first 2 days coincides with the biggest decrease in peak area (for unfrozen spraints) over the course of the study period. Conversely, no discernable effect can be seen due to rainfall prior to sampling on day 4, 8 and 16.

Between 8 - 16 days the 'ottery' odour of the outdoor spraint samples faded to reveal a more fish-like aroma (personal observation). Both Chapters 4 and 6 demonstrate the presence of suphur related compounds in otter scent marks. These chemicals may be in part responsible for the fish-like odour. This raises some important questions.

- If the spraints convey messages of otter individuality, then does diet have a role to play?

It has been shown that captive otters can distinguish the spraint odour of different individuals, irrespective of sex or level of previous experience (Trowbridge, 1983). In addition, all spraints from a particular individual can be recognised and associated. This was possible even though Trowbridge's test animals were consuming identical diets. Therefore, individual recognition cannot rely solely upon digestive by-products. It is possible that they could contribute to individuality; however there may be an alternative. Anal sac secretions are smeared onto passing faeces during deposition (Bradshaw et al, 2001). It is possible that the odour of the anal sac secretion masks that of the undigested food material. This might explain the fading of the spraint aroma to reveal the fish-like aroma beneath.

- How stable is spraint aroma in a natural setting?

If scent material is used for individual recognition, then it needs to be stable when placed in the environment for periods of time that at least match the frequency that a scent-mark will be encountered. If a scent mark is to be representative of an individual then it must possess this sort of stability (Albone & Perry, 1976; Albone et al, 1977; Preti et al, 1977).

The characteristic otter smell of the spraints was stable (to the human nose) over a period of somewhere between 8 and 16 days (personal observation). As previously mentioned, this may be due, in part for example, to the relative stability of P23.57 extracted using di-ethyl ether. It is known that hyaena pasting is still detectable by a human after 30 days – Mills, Gorman & Mills (1980). But how long does the 'otter odour' persist in the environment as a scent mark detectable by the olfactory system of an otter? How often does an individual return to replenish such a mark? If an otter habitually returned to spraint at a site before the previous mark had become ineffective then the scent would become a permanent feature (this would be important for both territorial maintenance and resource marking).

Otters have been shown to possess the ability to distinguish between their own old spraints and other old spraints even when 60 days old (a similar length of time that they persist in the field - Mason & Macdonald, 1986).

However, is this ability important in terms of territorial maintenance? Chanin (1993) states that wild otter males return to refresh scent-marks within their ranges every few days and that when they stop sprainting it takes less than a week for other otters to realise. If resident otters do indeed scent mark their ranges in such a rigid and regular way in order to protect it, this would mean that an otter might only have to find spraints that were a week old to decide that a range was no longer occupied. In other words, an otter may be *able* to recognise a 60 day old spraint, but this does not mean it this will be a viable scent mark in terms of territorial maintenance.

CONCLUSIONS

1. More than one approach may be needed for the analysis of otter spraints in relation to changes with length of time exposed to the environment. For example a solvent extraction coupled with headspace analysis.
2. Otter spraint material contains chemicals with opposite time dependence which could form the basis of a mechanism for pinpointing the age of a spraint (a useful tool both for human surveyors and the animals themselves).
3. Temperature / environmental conditions can have a significant effect on the rates of production and deterioration of chemicals within an otter spraint. This will affect accuracy of any estimate of age from spraints in the field (location, temperature, local weather conditions will all have to be taken into consideration).
4. In dry conditions, spraints undergo a significant loss in hydration in the first 2 days following deposition

FUTURE WORK

1. This study could be repeated in order to verify the result seen
2. GC-MS analysis would be needed in order to identify the important chemicals (with opposite time dependence)
3. The ability of otters to distinguish between spraints of differing age could be assessed as a comparison to similar studies previously carried out.

CHAPTER 4

SEXUAL DIFFERENCES IN THE ODOUR PROFILE OF EUROPEAN OTTER (*Lutra lutra*) SPRAINTS

INTRODUCTION

It has long been known that in many instances animal social organisation is regulated through olfactory signalling. 'Social odours' can provide important information regarding a signalling individual, e.g., information about species, age, sexual identity, individual identity, reproductive condition, group membership, kinship, familiarity, motivational state or environmental variables can all be conveyed in this way. (Buesching & Macdonald, 2004; Buesching et al, 2002b; Gueusi et al, 1996; Gorman, 1976; Hagey & Macdonald, 2003)

As discussed in Chapter 1, European otter, *Lutra lutra* sprainting behaviour has been linked with:

- Sexual behaviour;
- Territoriality;
- Resource marking;
- Individual identity

A limited number of studies have previously investigated the information found within European otter anal sac secretion using chemical analysis (Gorman et al, 1978; Bradshaw et al, 2001). Only one study has looked at spraint odour profiles (Trowbridge, 1983) but no elucidation of the chemical constituents was undertaken.

Identification of attractant and repellent compounds in spraints would be useful for possible manipulation of the behaviour of European otter populations/individuals, e.g., detering them from crossing roads known to be 'road-kill black spots' and enticing them into using specially built underpasses or prevention of fish predation at commercial fish farms.

Additionally, if the individuality or sexual code within the European otter spraint profiles could be deciphered revealing an individual odour 'fingerprint' and the age of a deposited spraint ascertained then this would prove an invaluable tool in monitoring both established otter populations and re-introduced or translocated animals, providing a far more accurate picture than simple spraint surveying.

In this study, the constituents of European otter scent mark material have been examined, initially by identifying those compounds present. Whilst other work of this

type has used anal gland secretion direct from the animal, this study used freshly deposited spraints, which included faecal material in addition to anal sac secretion. In this way, it was hoped to obtain the whole picture of the deposited scent mark odour (as an animal would encounter it in the wild). As a consequence the sampling process became as non-invasive as possible. The study used solid phase micro extraction (SPME) and gas chromatography – mass spectrometry (GCMS) to investigate the chemicals present in European otter spraints and whether any sex specific or individual patterns exist, either through unique sex specific / individual scent compounds or differing relative proportions of the same scent components.

MATERIALS AND METHODS

Study animals and sample collection.

3 sets of captive European otter spraints were collected from 3 separate otter sanctuaries (NB see Table 1 for more detailed information regarding the test animals and their diet):

- ***The Bowes Otter Sanctuary***, North Pennine Reserve, Bowes, County Durham, UK: spraint samples were collected from 6 captive-bred European otters, (3♂/3♀). Two spraint samples from each animal were collected on consecutive days.
- ***The Tamar Otter Sanctuary***, North Petherwin, Cornwall, UK: spraint samples were collected from 5 captive-bred European otters (2♂/3♀). Two spraint samples from each animal were collected on consecutive days.

Both the Bowes and the Tamar sanctuaries are run by The Otter Trust (The Otter Trust is the largest otter conservation body in the world and has been responsible for re-introductions of captive-bred otters into the eastern half of Britain since 1983)

- ***The Chestnut Centre***, Derbyshire, UK: spraint samples were collected from 6 captive-bred European otters (3♂/3♀). Three spraint samples from consecutive days were collected from each animal.

[The Chestnut Centre is a privately owned otter and owl sanctuary].

Spraint samples were collected 'immediately' following deposition – this was achieved by careful and patient observation of the animals. Each sample was placed individually into labelled airtight glass containers. The samples were stored over dry ice and immediately transferred to a -70°C freezer upon arrival at the laboratory to avoid deterioration prior to analysis.

NB Spraints were generally deposited near the corners and at the pen boundaries, although they were also found near the freshwater pools and lying-up shelters.

Table 1. Captive-bred European otter information

The Bowes Otter Sanctuary			
Pen	1	2	3
Otter	Flame	Wishful, Torridge & cub	Tilly & cub
Sex	male	female, male & male	female & female
Date of Birth	24/06/94	24/04/94, 12/06/94	23/10/91
History	captive-bred	captive-bred	captive-bred
The Tamar Otter Sanctuary			
Pen	1	2	3
Otter	Ripple & 2 cubs	Otty	Pebble
Sex	all female	male	female
Date of Birth	20/10/93, 10/01/00 + 10/01/00	12/95	26/07/96
Bowes & Tamar otter diet			
Feeding times	*Food*		
09:00 – 09:30	*dead, day old chicks*		
12:00 + 15:30	*filleted fish (variety of species) – 'token titbits' 7-8 ounces for public feeds*		
17:00 – 17:30	*ZF6 (Zoo Food – based on kitten food with rabbit, chicken and herbs, a complimentary, basic food for carnivorous wild animals). Adults eat half a tin (=200g) plus scraps (fish heads and more chicks). Extra eaten in winter – up to 3lb.*		
The Chestnut Centre			
Pen	1	2	3
Otter	Alpha & Izzy	Lancelot & Damsel	Morse & Sheil
Sex	male & female	male & female	male & female
Year of Birth	1994 & 1995	both 1997	1996 & 1997
Chesnut otter diet			
Feeding times	*Food*		
morning	*dead, day old chicks*		
afternoon	*fish (variety of species) + knackersmeat mixed with veg and a multivitamin (and sometimes oats). Suet given in winter. Also seen to catch rats, mice, bullheads.*		

NB all pens contained a freshwater pool for swimming.

Sample preparation and headspace analysis

Sub-samples were placed into 4ml amber glass vials (providing a consistent ratio of headspace : sample of 1:1). Volatile compounds were extracted (for 20 min at a constant temperature of 23°C) from the headspace using a solvent free solid phase micro-extractor (SPME) containing either a 65µm polydimethylsiloxane / divinylbenzene (PDMS/DVB) fibre or a 100µm polydimethylsiloxane (PDMS) fibre (Supelco, Bellefonte, PA). See Table 2 for further details:

Table 2. SPME fibre types used

Spraint sample set	Fibre type used
Bowes + Tamar	65µm polydimethylsiloxane/divinylbenzene (PDMS/DVB) -used for volatiles, amines and nitroaromatic compounds.
Chestnut	100µm polydimethylsiloxane (PDMS) -used for low molecular weight / volatile compounds

A different fibre type was used for the Chestnut spraint sample set. The 100µm PDMS fibre is more suited to extracting low molecular weight / volatile compounds and this was used to see whether the potentially different chemicals extracted would carry any similar messages to those extracted with the 65µm PDMS/DVB fibre.

GC analysis

Fibre contents were analysed by capillary GC-MS, with a CE Instruments HRGC 8000 Gas Chromatograph coupled with a Finnigan MD800 Mass Spectrometer, controlled by a Finnigan MD Family GC-MS Data System. The column was a DB-5MS column (J & W 30m, 0.316mm I.D., 0.25µm coating) with helium (1ml/min) as carrier gas.

Temperature programmes

The chromatograph was run for each extraction in a splitless mode (closed 2 min) and the following additional parameters: injector temperature 270ºC; detection temperature 300ºC; initial oven temperature 35ºC for 2 min; this was then increased by 5ºC/min up to 170ºC, this temperature was maintained for 2 min; then there was a further increase of 15ºC/min up to 270ºC, again maintained for 2 min; fibre desorption time was 5 min.

Relative retention times and peak fragmentation spectra obtained by GC-MS were analysed using Masslab (Finnigan, San José, CA, USA). Any peaks from the column/fibre (mainly siloxanes) and those from the empty vial control were discounted from the analysis. All substances were given tentative identification using the Finnigan MD Family GC-MS Data System NIST library (the best match was chosen in each case). NB: occasionally chemicals were identified as a type of

chemical (e.g., alkane / aldehyde). Where no close match could be found, the chemicals in question were labelled as '*??*'.

Total ion count (TIC) was used for each of the peaks on the resultant chromatograms, and these values were normalised to calculate the relative content of each compound in a sample. All peaks were used in the analyses.

Data transformations

Similarity coefficients such as the Bray-Curtis measure used here are sensitive to skewed data and in particular may be overly influenced by larger values which tend to dominate the resulting similarity coefficient. As a consequence some form of transformation is usually employed to downweight the influence of quantitatively dominant variables. A variety of transformations may be employed, depending on the level of transformation required, ranging from stronger transformations such as the log or root-root transformation (for datasets with a wide range of values including much larger values for some variables) to milder transformations such as the square root transformation. In the current study (and in studies in the following three chapters) a $\log_{10}$ transformation was employed for the raw/absolute data as this was heavily skewed.

Statistical data analysis

Multi- Dimensional Scaling (MDS) [using PRIMER 5 for Windows, version 5.2.0] was employed to clarify any underlying trends in the relatedness of compound composition. This enabled comparison of European otter spraint chemical profiles. The MDS analysis enabled comparison of:

- $\log_{10}$ transformation of 'absolute' content data;
- 'presence or absence' data

These analyses were performed on:

- Tamar sanctuary European otter spraint data alone
- Bowes sanctuary European otter spraint data alone
- Tamar and Bowes European otter spraint data combined
- Chestnut sanctuary European otter spraint data alone

The similarity percentage (SIMPER) routine, also using PRIMER (Clarke, 1993), was used to describe the relative importance of each chemical in terms of the similarity between the samples within, and between each group. SIMPER calculates the average Bray-Curtis 'similarity' between each pair of samples (individual otters) *within* each group (sex or sanctuary) and the average 'dissimilarity' between all pairs of samples between the groups (the individuals of group 1 against all individuals of group 2). The Bray-Curtis (dis)similarity measure between any pair of samples takes into account the relative contribution of each variable (chemical). Consequently, the similarity between individual samples within a group or the dissimilarity between individuals in each group can be explained with regard to the average contribution from each chemical constituent. These results highlight those chemicals which characterise each group and also those which discriminate between the groups. Chemicals that discriminate strongly between species will have a higher contribution to the inter-group dissimilarity between groups.

Analysis of similarities, or ANOSIM (Clarke and Green, 1988) was also carried out using the PRIMER software. This provided a way to test statistically whether there is a significant difference between two or more groups of sampling units (e.g., otter sex or sanctuary groups) based on the similarity matrix. A test statistic (R) is calculated, which defines the observed differences between groups in contrast to the differences within groups i.e. if males were very dissimilar to females the differences in average rank similarities between the male and female groups would be much higher than those within each group. In order to test these differences the R statistic is recomputed under permutations of the sample labels whereby the labels which define which sample belongs to which group are then randomly rearranged and the R value recalculated. This is repeated for the desired number of permutations. Therefore, a big difference between male and female groups would give a high R value in the original statistic so when the data is shuffled randomly for a given number of permutations the number of times a permuted R value is calculated, which is as big as the actual R value is likely to be relatively low – the significance level of the test is then calculated by referring the observed R value to its permutation distribution. If there was little difference between groups the observed global R value will be low and therefore random values of R under permutation are likely include a greater number of R values as high as the observed value so the significance will be low.

In addition, in comparing the spraint profiles, the concept of `overlap' between samples was used. This is defined as the number of compounds common to both samples as a percentage of the *total* number of compounds in each individual sample. For instance, if two otters have 15 compounds in common between their respective samples, and the first otter has a total of 20 compounds present in his samples and the second otter has 15, then the overlap for otter 1 will be 75% and the overlap for otter 2 will be 100%.

The differences found between samples were tested for significance using independent *t*-tests. Levene's test for equality of variances was initially employed to identify the most appropriate *t*-test (equal variances assumed/equal variances not assumed).

RESULTS

Volatiles and semi-volatiles obtained directly (using SPME) from captive-bred *L lutra* spraint material were analysed.

Tamar Sanctuary: 104 separate compounds were found using a 65µm polydimethyl-siloxane/divinylbenzene (PDMS/DVB) fibre - for volatiles, amines and nitroaromatic compounds (see Table 3)

Bowes Sanctuary: 121 separate compounds were found using a 65µm polydimethyl-siloxane/divinylbenzene (PDMS/DVB) fibre - for volatiles, amines and nitroaromatic compounds (see Table 4)

Table 3. Chemical compounds in European otter spraints from the Tamar sanctuary.

Alkanes/Alkenes
6-methyl-1-heptene *(2.667)*
C9 *(3.117)*
3-carene *(3.668)*
7-methyl-6-tridecene *(4.101)*
3,5,5-trimethyl-2-hexene *(4.418)*
(E)-5(pentyloxy)-2-pentene *(4.684)*
C10 *(5.035)*
form of cyclohexane *(6.702)*
pentyl cyclopropane *(6.735)*
2-ethyl-1,1-dimethyl-cyclopentane *(7.402)*
C11 *(7.519)*
3,3-dimethyl-1-pentene *(7.852)*
6-methyl 4-undecene *(8.686)*
1-methyl-2-pentyl-cyclopropane *(9.486)*
naphthalene *(9.669)*
6-ethyl-2-methyl-decane *(10.268)*
decane, 2,3,5-trimethyl- *(13.087)*
1-heptadecene *(14.154)*
form of cyclohexane *(15.721)*
17-pentatriacontene *(16.688)*
form of cyclohexadiene *(17.238)*
docosane *(17.388)*
2,6,10,14-tetramethyl-heptadecane *(18.422)*
alkane *(20.906)*
4A,8A-butano 1,4 dioxino 2,3-B - 1,4-dioxin, tetrahydro
3-bromo-decane *(23.273)*
C13 *(23.373)*
heptadecane, 2,6,10,15-tetramethyl- *(25.524)*
alkane *(25.641)*
pentatriacontane *(32.026)*
alkane *(33.893)*
alkane *(35.193)*
alkane *(36.243)*

Ketones
2-tridecanone *(2.934)*
6-methyl-2-heptanone *(4.034)*
6-methyl-5-hepten-2-one *(4.668)*
2,2,6-trimethyl-cyclohexanone *(5.802)*
3-heptanone, 2,4-dimethyl *(6.035)*
2-undecanone *(7.252)*
6,10-dimethyl-2-undecanone *(10.036)*
6,7-dodecanedione *(11.353)*
4-undecanone *(12.287)*
2-tridecanone *(12.854)*
6,10-dimethyl-2-undecanone *(15.838)*
cis-geranylacetone *(16.988)*
9-decen-2-one *(18.255)*
2(4H)-benzofuranone, 5,6,7,7A-tetrahydro- 4,4,7H-trimethyl-, (R)- *(18.839)*
2(3H)-furanone, dihydro-5-octyl *(31.792)*

Aldehydes
(E)-2-octenal *(2.5)*
benzaldehyde *(4.184)*
octanal *(5.085)*
benzeneacetaldehyde *(6.018)*
(E)-2-octenal *(6.385)*
nonanal *(7.619)*
1-nonanal (decyl ester) *(8.852)*
(E,Z)-2,6-nonadienal *(8.919)*
trans-2-undecanal *(9.119)*
2-isopropyl-5-oxohexanal *(10.036)*
decanal *(10.436)*
trans-2-undecanal *(11.987)*
trans-2-undecanal *(12.003)*
2,4-decadienal *(13.52)*
dodecanal *(16.038)*
tridecanal *(18.672)*
tetradecanal *(21.189)*
aldehyde *(23.573)*
octadecanal *(25.857)*

Alcohols
heptanol *(4.368)*
phenol *(4.518)*
1-hepten-3-ol *(4.584)*
2-ethyl-1-hexanol *(5.668)*
benzyl alcohol *(5.768)*
trans-2-undecan-1-ol *(6.635)*
L-1,2,3-trimethylbicyclo 2.21 -2-heptanol *(7.152)*
phenylethyl alcohol *(7.769)*
4,8-dimethyl 1-nonanol *(8.386)*
bicyclo 2.2.1 heptan-7-ol *(12.137)*
cyclohexaneethanol, acetate *(12.37)*
2-decanol *(13.17)*

Carboxylic Acids
ethaneperoxic acid *(7.685)*
2-methyl-2-propenoic acid *(8.269)*
form of butanoic acid *(10.754)*
form of propanoic acid *(14.955)*
tetradecanoic acid *(24.724)*
hexadecanoic acid *(29.025)*
eicosanoic acid *(36.094)*

Benzene compounds
(1-methylethyl)-benzene *(3.484)*
1,4-dichlorobenzene *(5.301)*
diethyl benzene *(6.302)*
form of benzene *(7.018)*

Nitrogen compounds
indole *(12.704)*
5-acetyl-2-methylpyridine *(12.92)*
decanamide, N,N-diethyl *(24.04)*

Sulphur compounds
dimethyl trisulfide *(4.334)*
1,1'-sulfonylbis 4-chloro-benzene *(34.176)*

Others
furan-2-propyl *(5.268)*
pentyl-3-methylbutanoate *(7.702)*
a-cyclocitral *(10.703)*
?? *(22.607)*
(E,E)-farnesol *(26.124)*
bis(2-methoxyethyl)phthalate *(26.674)*
?? *(27.241)*
bis(2-methoxyethyl)phthalate *(28.658)*
phenol, 4,4'-(1-methylethylidene)bis- *(33.376)*
bis(2-ethylhexyl)phthalate *(37.344)*

Table 4. Chemical compounds in European otter spraints from the Bowes sanctuary.

Alkanes/Alkenes
3-methyl-tridecane *(1.109)*
1-tert-butoxy-2-methoxyethane *(1.393)*
6-methyl-1-heptene *(3.21)*
3-carene *(4.427)*
7-methyl-6-tridecene *(4.994)*
(E)-5(pentyloxy)-2-pentene *(5.744)*
C10 *(6.127)*
2,6-dimethyl-2-trans-6-octadiene *(7.511)*
form of cyclohexane *(8.062)*
pentyl cyclopropane *(8.128)*
propyl-thiopane *(8.562)*
C11 *(8.945)*
6-methyl 4-undecene *(10.262)*
oxirane, tetradecyl- *(10.929)*
1-methyl-2-pentyl-cyclopropane *(11.079)*
naphthalene *(11.163)*
6-ethyl-2-methyl-decane *(11.879)*
decane, 2,3,5-trimethyl- *(14.747)*
1-heptadecene *(15.814)*
form of cyclohexane *(17.414)*
2,6,10,14-tetramethyl-heptadecane.*(20.099)*
alkane *(22.583)*
3-bromoodecane *(24.934)*
C13 *(25.027)*
cyclopentane, (2-hexyloctyl)- *(25.834)*
heptadecane, 2,6,10,15-tetramethyl- *(27.201)*
alkane (branched) *(27.318)*
heptadecane, 2,6,10,15-tetramethyl- *(29.335)*
(E,E)-7,11,15-trimethyl-3-methylenehexadeca-1,6,10,14-tetraene *(29.485)*
heptadecane, 2,6,10,15-tetramethyl- *(31.403)*
pentatriacontane *(33.52)*
hexadecane, 1-(ethenyloxy)- *(34.437)*
alkane *(35.621)*
alkane *(36.955)*
alkane *(38.022)*
alkane *(38.972)*

Ketones
2-azetidinone, 3,4,4-trimethyl- *(1.643)*
2H-pyran-2-one, tetrahydro-4-methyl-. *(1.876)*
2-tridecanone *(3.543)*
6-methyl-5-hepten-2-one *(5.709)*
2,2,6-trimethyl-cyclohexanone *(6.995)*
3-heptanone, 2,4-dimethyl- *(7.328)*
2-undecanone *(8.662)*
3,4,4-trimethyl-2cyclohexen-1-one *(9.479)*
6,7-dodecanedione *(12.96)*
4-undecanone *(13.93)*
2-tridecanone *(14.514)*
2-butanone, 4(5-methyl-2-furanyl)- *(17.448)*
6,10-dimethyl-2-undecanone *(17.515)*
cis-geranylacetone *(18.682)*
9-decen-2-one *(19.932)*
2(4H)-benzofuranone, 5,6,7,7A-tetrahydro- 4,4,7H-trimethyl-, (R)- *(20.498)*
4H-1-benzopyran-4-one, 2-(3,4-dimethoxyphenyl)-3,5-dihydroxy-7-methoxy- *(24.951)*
cyclopentadecanone, 2-hydroxy- *(30.512)*
2(3H)-furanone, dihydro-5-octyl- *(33.353)*

Aldehydes
heptanal *(3.793)*
benzaldehyde *(5.077)*
octanal *(6.211)*
benzeneacetaldehyde *(7.228)*
(E)-2-octenal *(7.695)*
(E)-4-nonenal *(8.845)*
nonanal *(9.062)*
(E,Z)-2,6-nonadienal *(10.429)*
trans-2-undecanal *(10.646)*
2-isopropyl-5-oxohexanal *(11.629)*
(E)-4-nonenal *(11.663)*
decanal *(12.03)*
trans-2-undecanal *(13.613)*
2,4-decadienal *(15.181)*
dodecanal *(17.715)*
tridecanal *(20.349)*
tetradecanal *(22.867)*
aldehyde *(25.251)*
octadecanal *(27.535)*
(Z)-9-octadecenal *(29.152)*
aldehyde *(29.702)*
aldehyde (*31.786*)

Alcohols
cyclopentanol, 2-methyl-, trans- *(2.21)*
heptanol *(5.377)*
1-hepten-3-ol *(5.611)*
phenol *(5.627)*
2-ethyl-1-hexanol *(6.927)*
benzyl alcohol *(7.011)*
trans-2-undecan-1-ol *(7.995)*
phenylethyl alcohol *(9.229)*
bicyclo 2.2.1 heptan-7-ol *(13.78)*
cyclohexaneethanol, acetate *(13.997)*
cyclohexaneethanol, acetate *(14.147)*
phenol, 4,4'-(1-methylethylidene)bis- *(35.22)*
6,10,14-hexadecatrien-1-ol, 3,7,11,15-tetramethyl-, R-(E,E)- *(36.855)*

Carboxylic acids
butanoic acid, 3-methyl- *(2.81)*
2,6,10-dodecatrienoic acid, 3,7,11-trimethyl, methyl ester (E,Z)- *(8.195)*
form of butanoic acid *(12.879)*
form of propanoic acid *(16.581)*
tetradecanoic acid *(26.368)*
hexadecanoic acid *(30.636)*
2-propenoic acid, 3-(4-methoxyphenyl_-2-ethylhexyl ester *(34.754)*
tetradecanoic acid, octadecyl ester *(36.304)*
2-propenoic acid, 3-(4-methoxyphenyl_-2-ethylhexyl ester *(37.071)*
eicosanoic acid *(37.705)*

Nitrogen compounds
indole *(14.364)*
5-acetyl-2-methylpyridine *(14.562)*
pyrazine, 2,3-dihydro-5,6-di-2-pyridinyl- *(23.017)*
decanamide, N,N-diethyl *(25.717)*
n-nitroso-2-isopropyl-4,4-dimethyloxazolidine *(37.905)*

Sulphur compounds
1,1'-sulfonylbis 4-chloro-benzene *(35.904)*

Benzene compounds
benzene, 1(1-ethylnonyl)- *(18.949)*

Others
?? *(3.577)*
furan-2-propyl *(6.394)*
a-cyclocitral *(12.263)*
4A,8A-butano 1,4 dioxino 2,3-B - 1,4-dioxin, tetrahydro *(23.133)*
xylofuranose *(23.75)*
?? *(24.267)*
isopropyl myristate *(27.718)*
(E,E)-farnesol *(27.801)*
bis(2-methoxyethyl)phthalate *(28.335)*
?? *(28.902)*
bis(2-methoxyethyl)phthalate *(28.335)*
?? *(33.22)*
butyl, hexadecanoate *(35.354)*
bis(2-ethylhexyl)phthalate *(39.205)*

NB Both Table 3 and Table 4 are arranged according to compound class, although some may fall into more than one class. Chemical compounds (121) in total isolated from European otter spraint material and used in the statistical analysis. Known compounds (checked against standard chemicals) are highlighted (**blue**) together with straight chain alkanes and aldehydes (*italics*). All other compounds are tentatively identified using Finnigan MD Family GC-MS Data System NIST library.

A variety of organic compounds were found within the SPME extractions from both the Tamar and the Bowes sanctuary spraint samples (there was a high degree of overlap observed between the two sample sets). These compounds included e.g., alkanes, alkenes, aldehydes, alcohols, phthalates, furanones. Many of these chemicals have been identified in the scent marks of other species, both mustelid and non-mustelid. (Examples of the gas chromatograms produced from males and females from both sanctuaries can be seen in Figures 1-4).

Chestnut Sanctuary: 19 separate compounds were found using a 100µm polydimethylsiloxane (PDMS) – for low molecular weight / volatile compounds fibre - for volatiles, amines and nitroaromatic compounds (see Table 5)

Table 5. Chemical compounds in European otter spraints from the Bowes sanctuary.

cyclobutanol	*C6*	dihydro-3,5-dimethyl-2(3H)-furanone
isopropyl alcohol	chloroform	ethyl benzene
tetramethyloxirane	trans-7-methyl-3-octene	styrene
C4	3-methyl-butanal	**benzaldehyde**
methylene chloride	hydroxylamine	(E)-2-heptenal
o-(2-methylpropyl)-hydroxylamine	2,2-dimethyl hexane	
3-ethyl-2,2-dimethyl-pentane	2-chloro-2-nitro-propane	

Known compounds (checked against standard chemicals) are highlighted (**blue**) together with straight chain alkanes and aldehydes (*italics*).

Far fewer organic compounds were found within the SPME extractions (using the alternative type of fibre) from the Chestnut sanctuary spraint samples. These compounds included e.g., alkanes, alkenes, aldehydes, alcohols, phthalates, furanones.

Examples of the gas chromatograms produced from males and females from the Chestnut sanctuary can be seen in Figures 5 and 6.

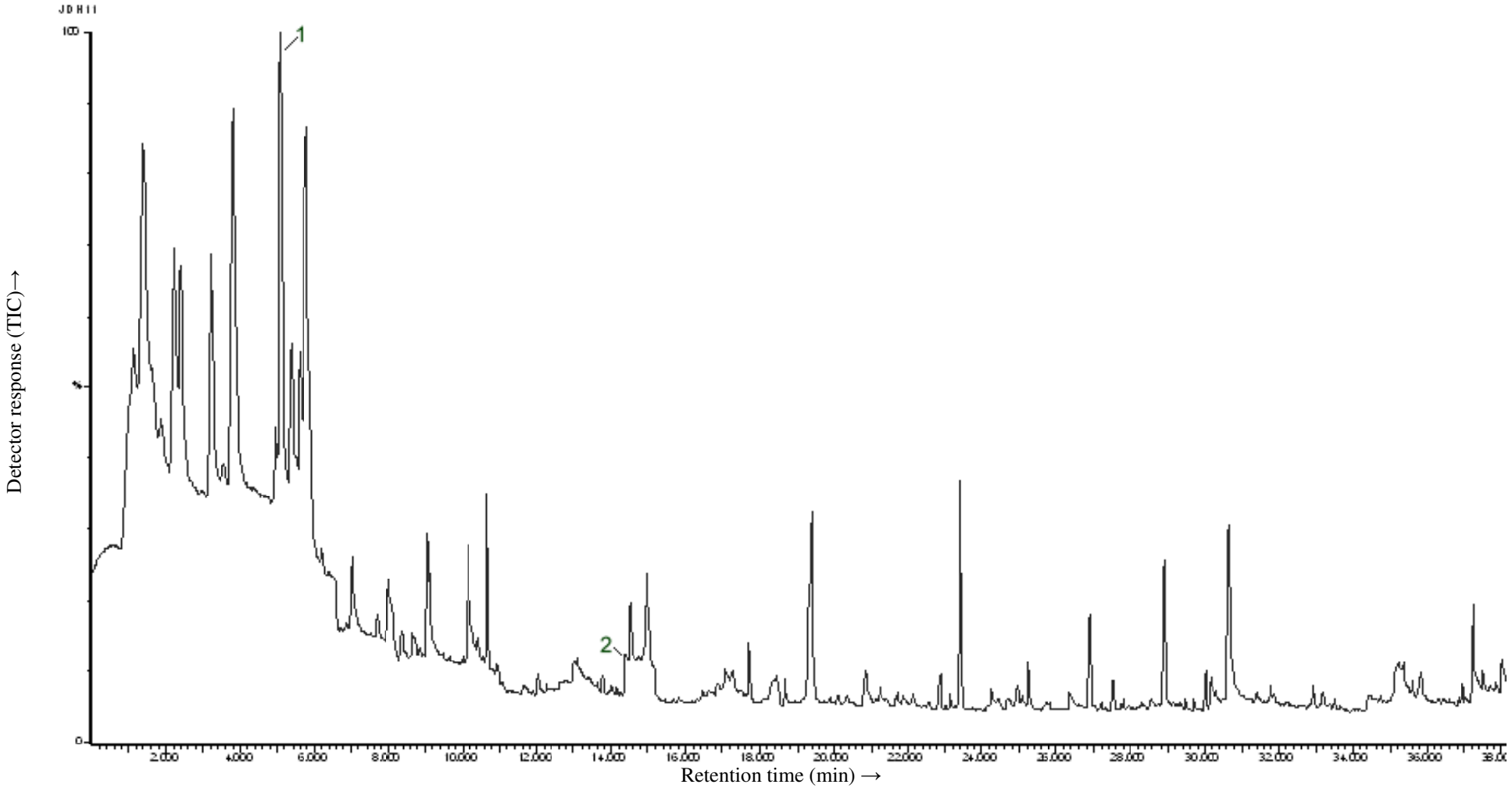


Figure 1. Gas chromatogram showing SPME extractions of a MALE captive European otter spraint samples from the BOWES sanctuary. Known compounds (checked against standard chemicals) – 1: benzaldehyde / 2: indole.

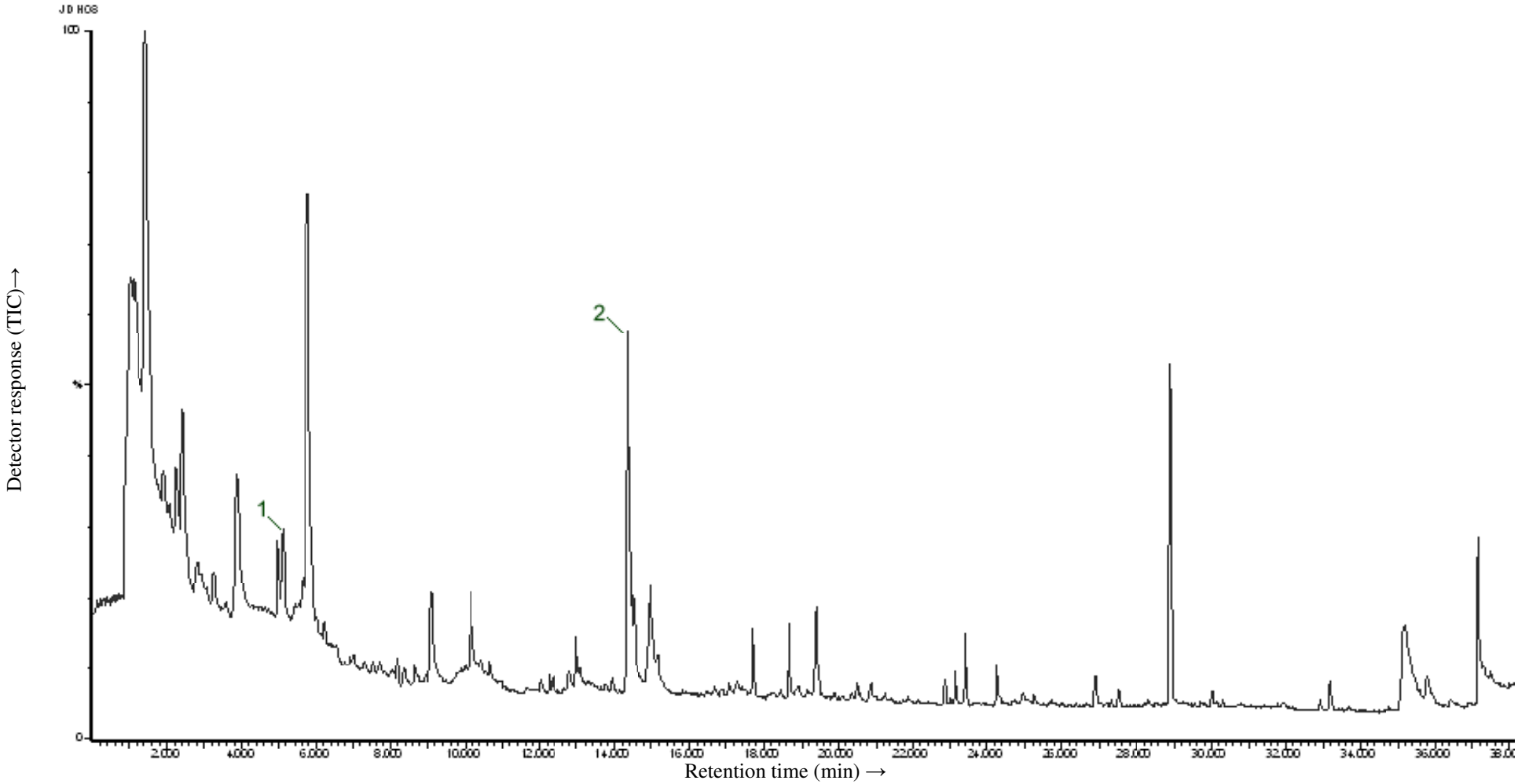


Figure 2. Gas chromatogram showing SPME extractions of a FEMALE captive European otter spraint samples from the BOWES sanctuary. Known compounds (checked against standard chemicals) – 1: benzaldehyde / 2: indole.

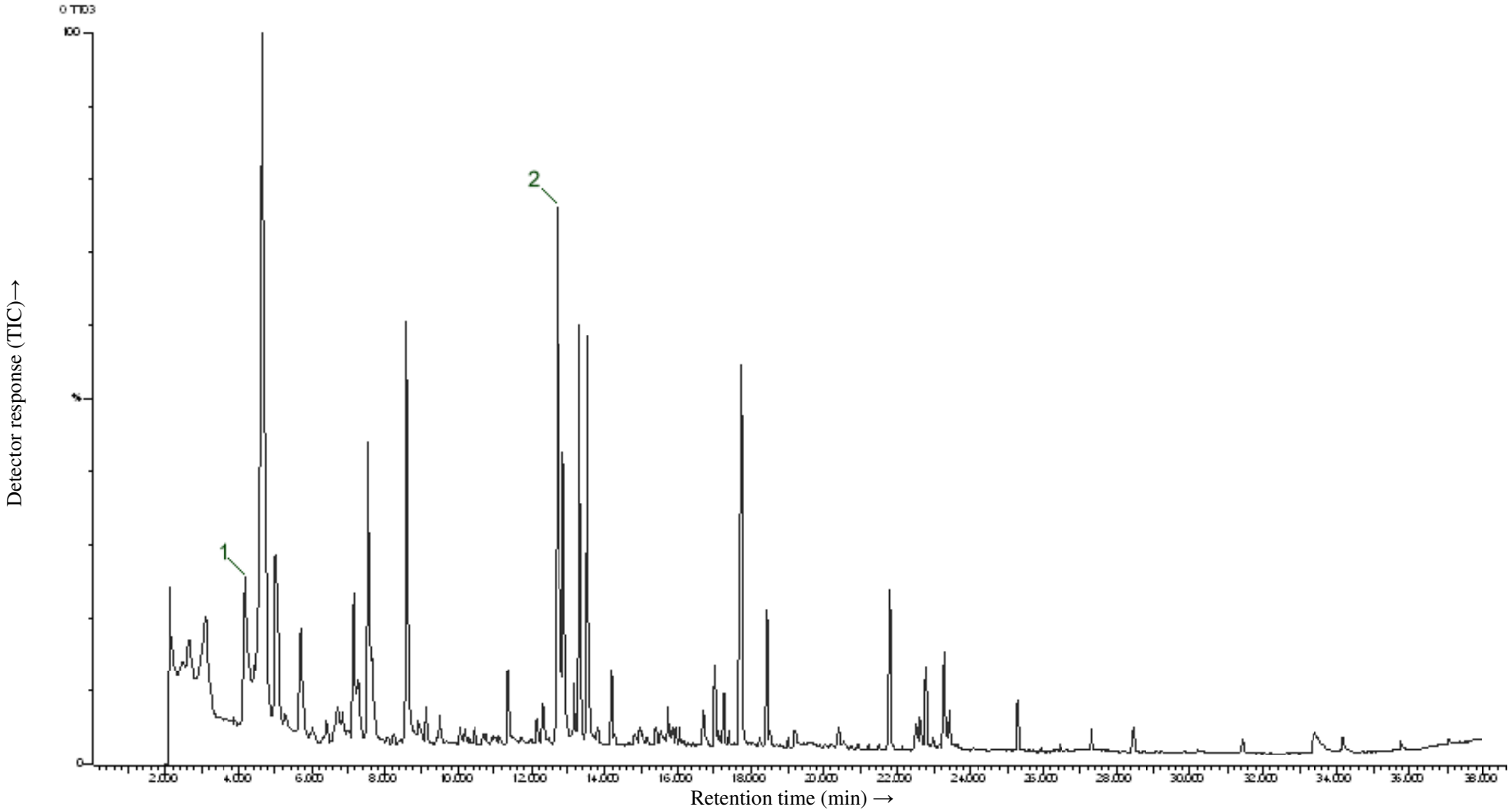


Figure 3. Gas chromatogram showing SPME extractions of a MALE captive European otter spraint samples from the TAMAR sanctuary. Known compounds (checked against standard chemicals) – 1: benzaldehyde / 2: indole.

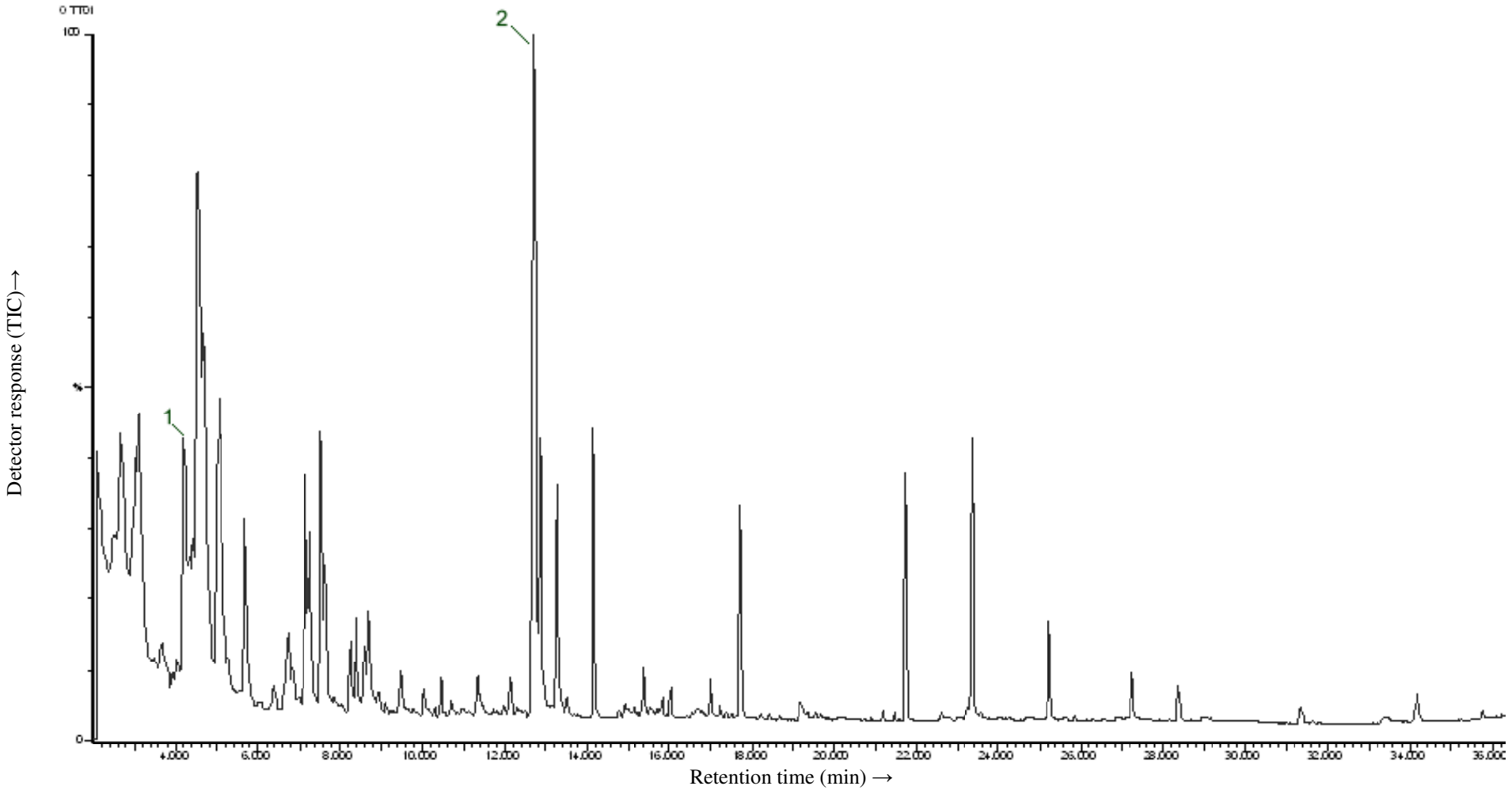


Figure 4. Gas chromatogram showing SPME extractions of a FEMALE captive European otter spraint samples from the TAMAR sanctuary. Known compounds (checked against standard chemicals) – 1: benzaldehyde / 2: indole.

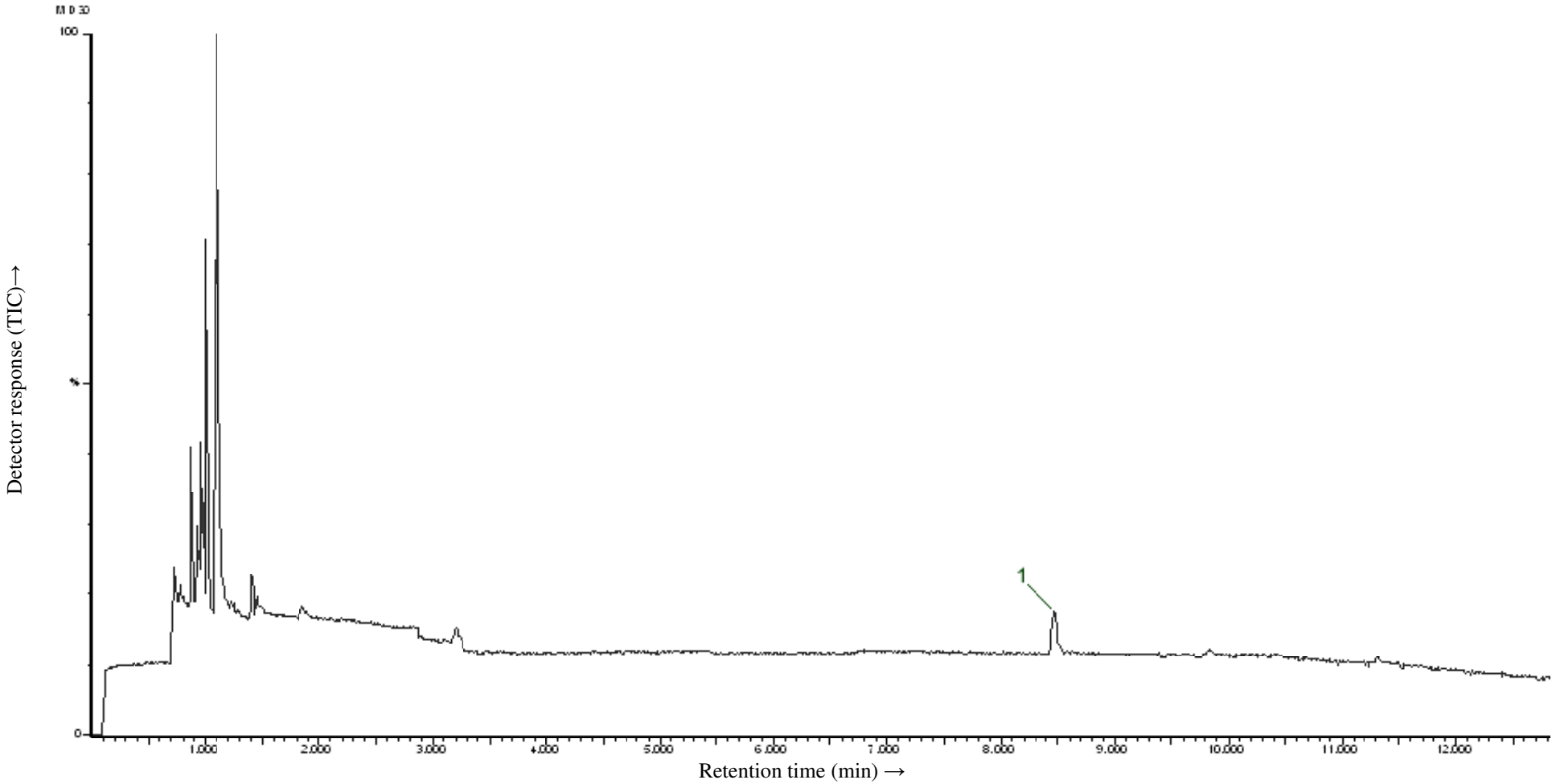


Figure 5. Initial section of gas chromatogram showing SPME extractions of a MALE captive European otter spraint samples from the CHESTNUT sanctuary. Known compound (checked against standard chemicals) – 1: benzaldehyde

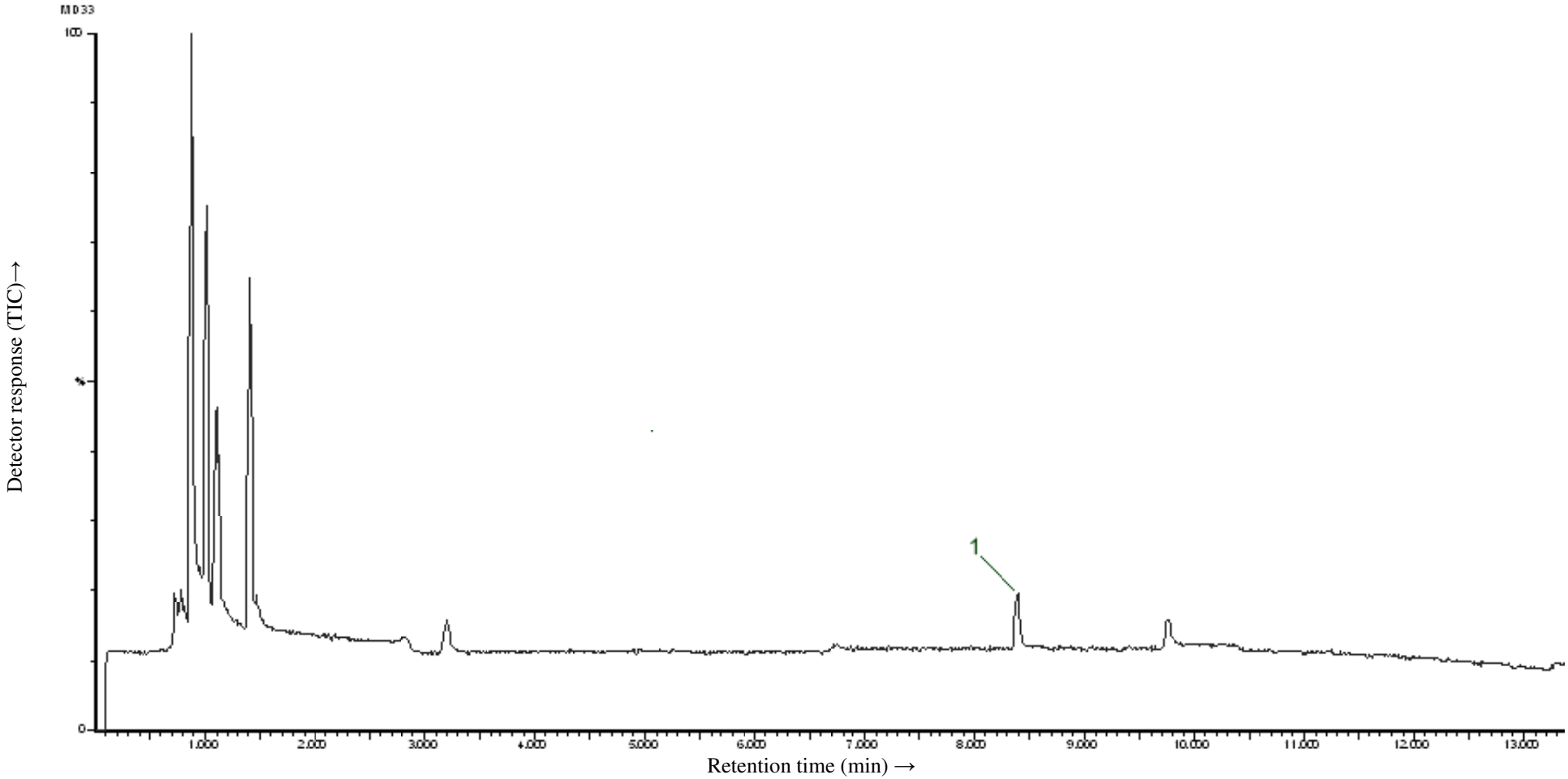


Figure 6. Initial section of gas chromatograms showing SPME extractions of a FEMALE captive European otter spraint samples from the CHESTNUT sanctuary. Known compound (checked against standard chemicals) – 1: benzaldehyde

Comparisons of sanctuaries, sexes and individuals

Overlap in compound composition within and between the sex groups in captive otter spraints from the Tamar otter sanctuary alone

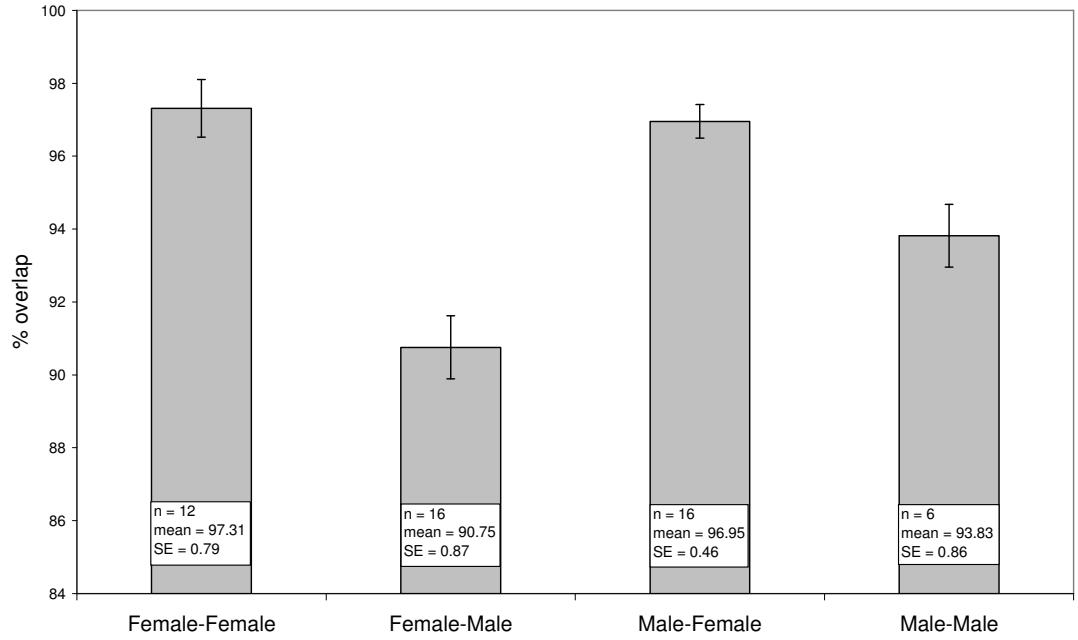


Figure 7. Overlap in compound composition within and between the sex groups in captive European otter spraints from the Tamar otter sanctuary alone. ['n' refers to the 'number of comparisons' in the overlap analysis]

Figure 7 shows the difference in compound overlap between the sexes (using data from the Tamar otter sanctuary alone). The mean overlap within the female group was higher than the mean overlap between females and males. In contrast the mean overlap within the male group was lower than the mean overlap between males and females. The levels of significance of these differences were calculated using an independent *t*-test. All differences were statistically significant. See Table 5.

Table 5. Comparison of overlap in spraint odour compound composition within and between Tamar European otter sex groups – independent t-test results.

Comparison	*t value*	*df*	*Sig.(2-tailed)*
Male-Male vs *Male-Female*	-3.420	20	<0.005
Female-Female vs *Female-Male*	5.410	26	<0.005

Overlap in compound composition within and between the sex groups in captive otter spraints from the Bowes otter sanctuary alone

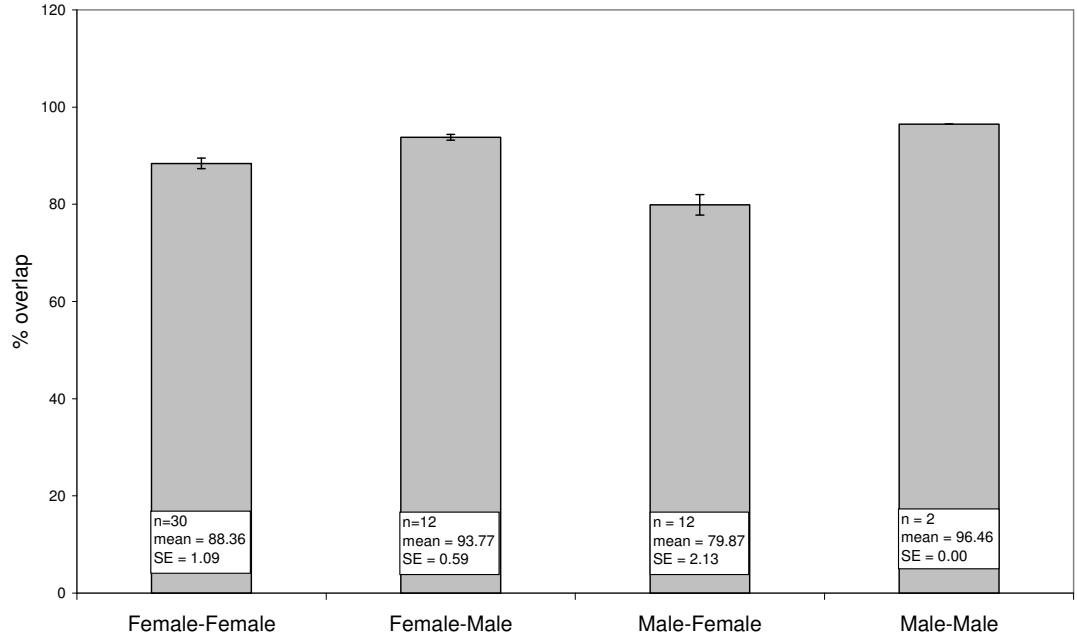


Figure 8. Overlap in compound composition within and between the sex groups in captive European otter spraints from the Bowes otter sanctuary alone. ['n' refers to the 'number of comparisons' in the overlap analysis]

Figure 8 shows the difference in compound overlap between the sexes (using data from the Bowes otter sanctuary alone). The mean overlap within the female group was lower than the mean overlap between females and males. In contrast the mean overlap within the male group was higher than the mean overlap between males and females. The levels of significance of these differences were calculated using an independent *t*-test. All differences were statistically significant. See Table 6.

Table 6. Comparison of overlap in spraint odour compound composition within and between Bowes European otter sex groups – independent t-test results.

Comparison	*t value*	*df*	*Sig.(2-tailed)*
Male-Male vs *Male-Female*	7.792	11	<0.005
Female-Female vs *Female-Male*	4.328	39.533	<0.005

Overlap in compound composition within and between the sex groups in captive otter spraints from both Tamar and Bowes otter sanctuaries

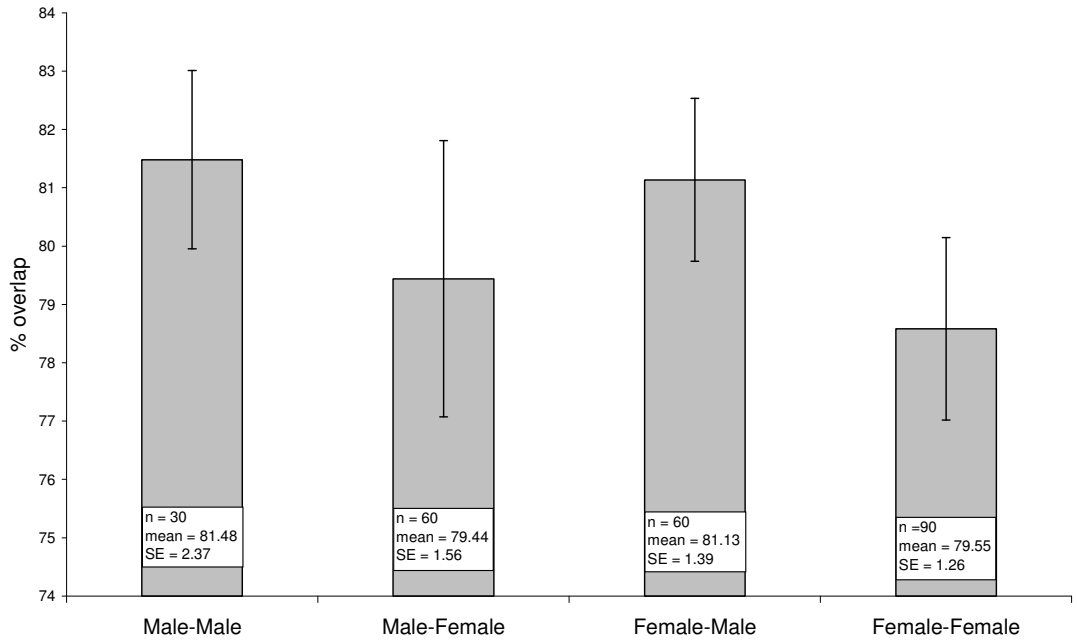


Figure 9**.** Overlap in compound composition within and between the sex groups in captive European otter spraints from both Tamar and Bowes otter sanctuaries. ['n' refers to the 'number of comparisons' in the overlap analysis]

Figure 9 shows the difference in compound overlap between the sexes (using data from both the Tamar and the Bowes otter sanctuaries). The mean overlap within the male group was higher than the mean overlap between males and females. In contrast the mean overlap within the female group was *lower* than the mean overlap between females and males. The levels of significance of these differences were calculated using an independent *t*-test. None of the differences were statistically significant. See Table 7.

Table 7. Comparison of overlap in spraint odour compound composition within and between Tamar and Bowes European otter sex groups – independent t-test results.

Comparison	*t value*	*df*	*Sig.(2-tailed)*
Male-Male vs *Male-Female*	0.736	88	0.464
Female-Female vs *Female-Male*	-0.825	148	0.411

Overlap in compound composition within and between the different sanctuary groups (Tamar and Bowes) in captive otter spraints

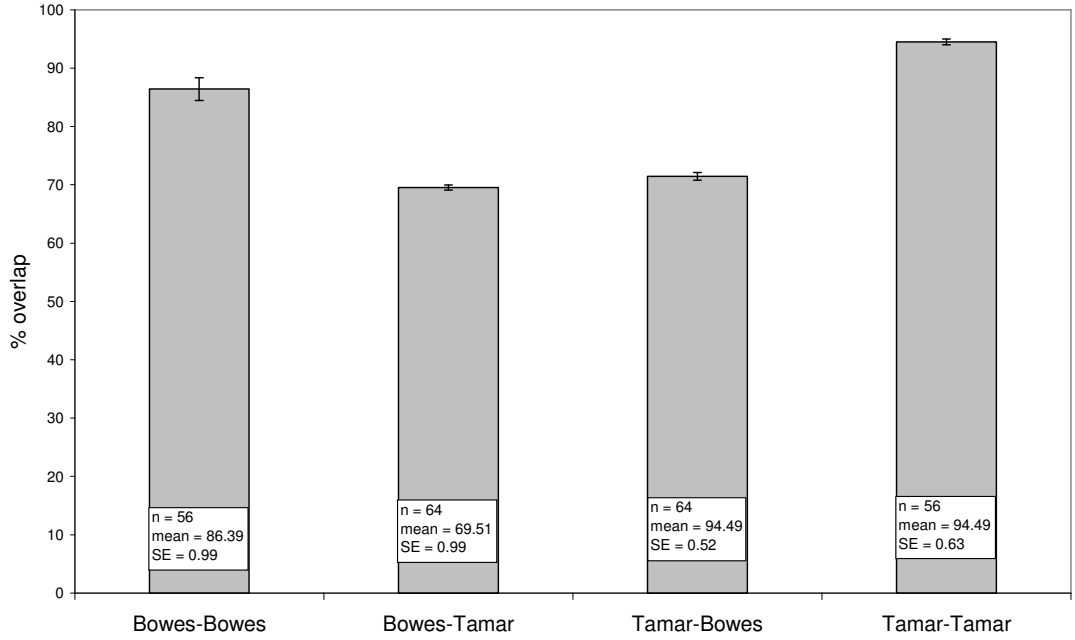


Figure 10. Overlap in compound composition within and between the different sanctuary groups (Tamar and Bowes) in captive European otter spraints. ['n' refers to the 'number of comparisons' in the overlap analysis]

Figure 10 shows the difference in compound overlap between spraints from different sanctuaries (Tamar and Bowes). The mean overlap within the Bowes group was higher than the mean overlap between the Bowes group and the Tamar group. Similarly, the mean overlap within the Tamar group was higher than the mean overlap between the Tamar group and the Bowes group. The levels of significance of these differences were calculated using an independent *t*-test. All differences were statistically significant. See Table 8.

Table 8. Comparison of overlap in spraint odour compound composition within and between Tamar and Bowes European otter sanctuary groups – independent t-test results.

Comparison	*t value*	*df*	*Sig.(2-tailed)*
Bowes -Bowes vs *Bowes-Tamar*	16.943	76.328	<0.005
Tamar-Tamar vs *Tamar-Bowes*	27.795	118	<0.005

Overlap in compound composition within and between the sex groups in captive otter spraints from the Chestnut otter sanctuary alone

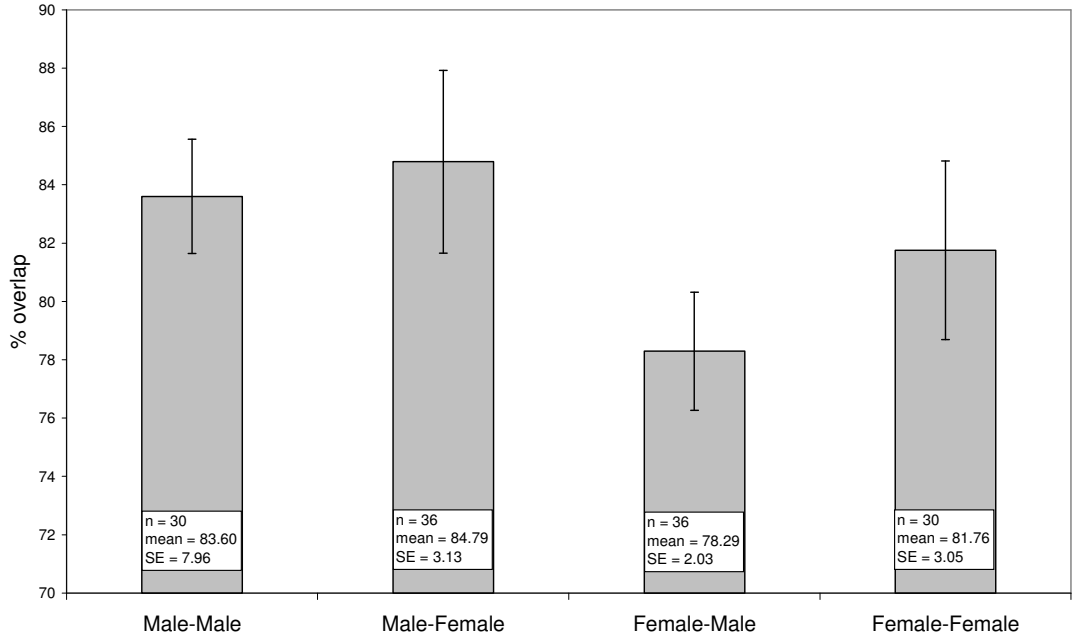


Figure 11. Overlap in compound composition within and between the sex groups in captive European otter spraints from the Chestnut otter sanctuary alone. ['n' refers to the 'number of comparisons' in the overlap analysis]

Figure 11 shows the difference in compound overlap between the sexes (using data from the Chestnut otter sanctuary alone). The mean overlap within the Male group was lower than the mean overlap between males and females. In contrast the mean overlap within the female group was higher than the mean overlap between males and females. The levels of significance of these differences were calculated using an independent *t*-test. None of the differences were statistically significant. See Table 9.

Table 9. Comparison of overlap in spraint odour compound composition within and between Chestnut European otter sex groups – independent t-test results.

Comparison	*t value*	*df*	*Sig.(2-tailed)*
Male-Male vs *Male-Female*	-0.306	64	0.761
Female-Female vs *Female-Male*	-0.972	64	0.335

Comparison of spraints from Tamar sanctuary *European otter* individuals using Multi-Dimensional Scaling (MDS)

Absolute content data, log_{10} transformed 1 way ANOSIM [R=0.81, p=0.029]

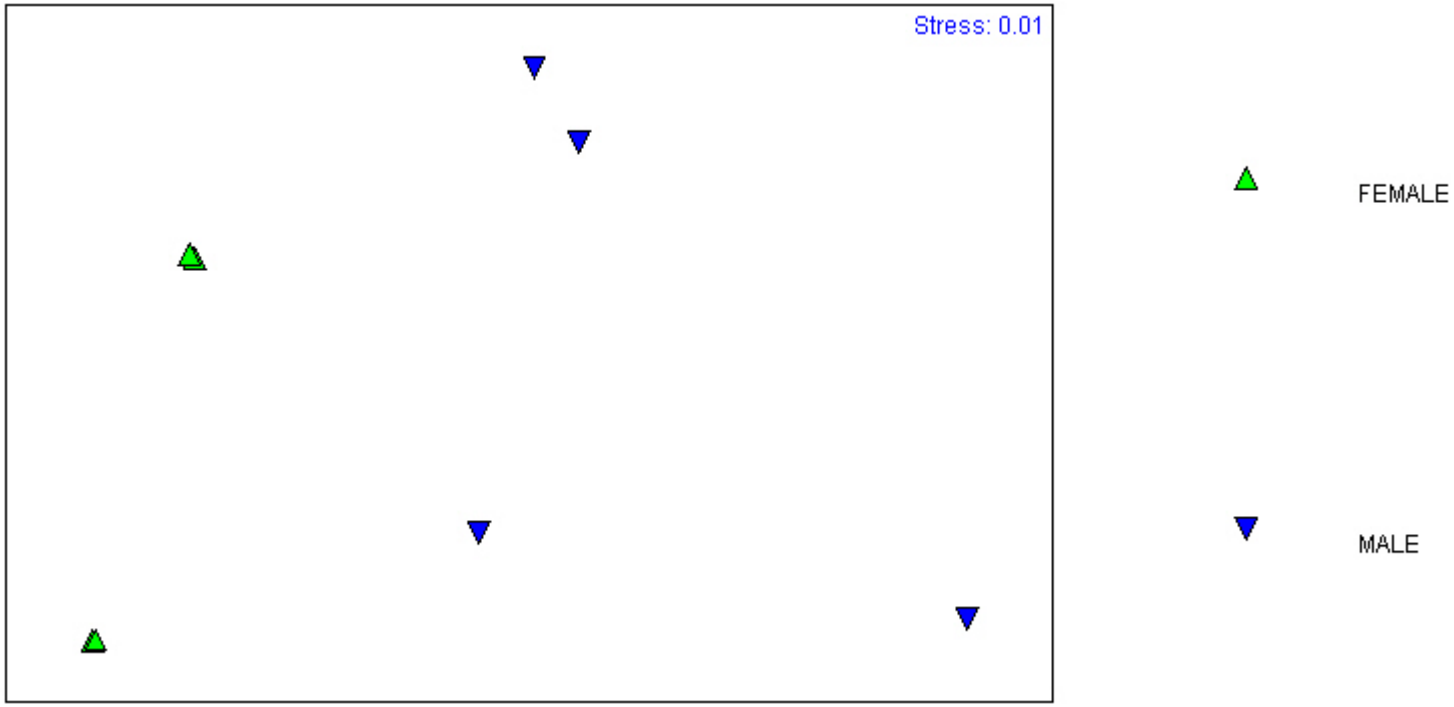


Figure 12. Multi Dimensional Scaling diagram showing relatedness between Tamar European otter sexes, based on log_{10} transformed absolute scent mark odour chemical data. ANOSIM R values indicate significant differences between the sexes.

Presence or absence data 1 way ANOSIM [R=0.73, p=0.029]

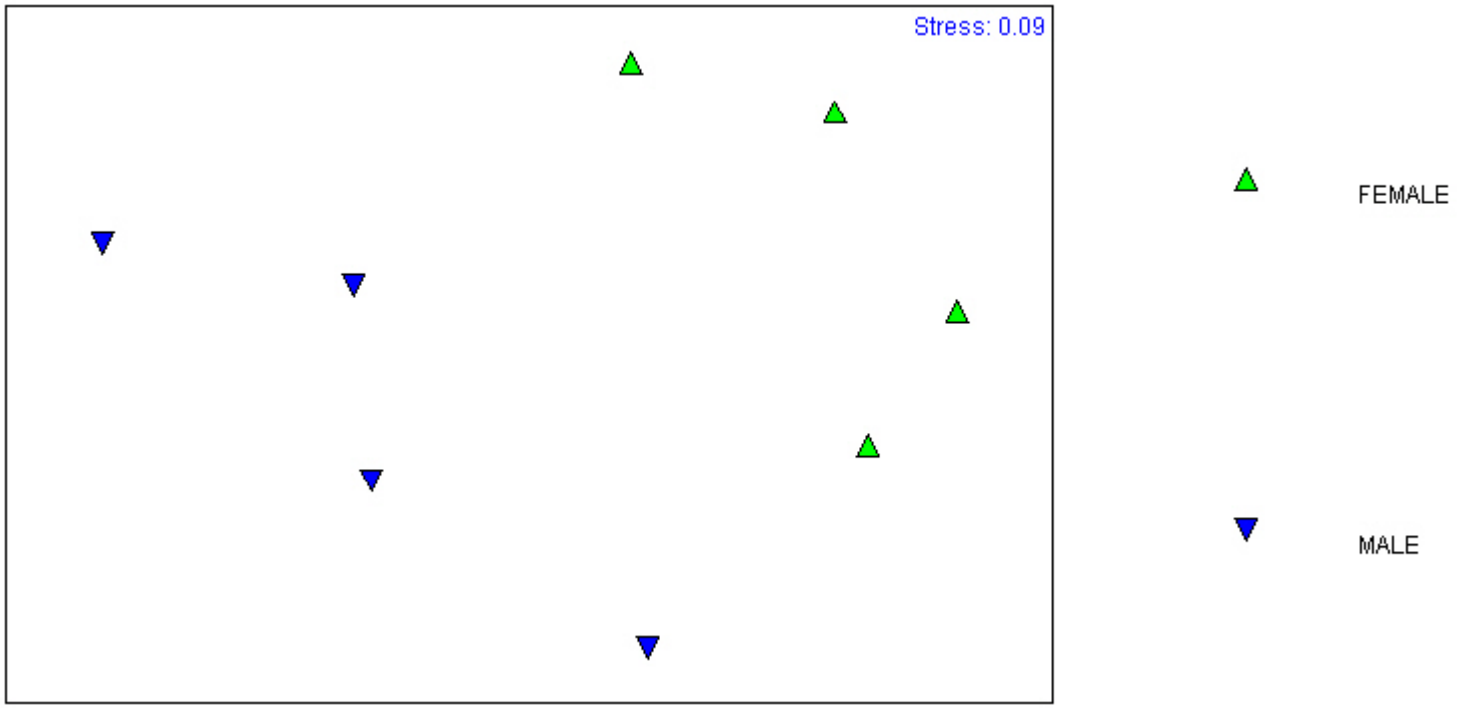


Figure 13. Multi Dimensional Scaling diagram showing relatedness between Tamar European otter sexes, based on presence or absence scent mark odour chemical data. ANOSIM R values indicate significant differences between the sexes.

Both MDS and ANOSIM analyses using spraint odour chemical data (absolute and presence or absence) showed a similar result; a clear separation of Tamar sanctuary sex groups. ANOSIM R values indicate significant differences between the sexes for both data types.

Further analysis was now carried out in order to ascertain which chemicals might be most responsible for the separation seen. Table 10 summarises the % similarity within the sex groups, plus the chemicals most responsible for these similarities. Table 11 summarises and the dissimilarity between the sex groups for each data type plus the chemicals most responsible for these dissimilarities.

SIMPER analysis

Similarity / dissimilarity summary tables

Table 10. Average percentage similarity (plus spraint chemicals with the largest contribution to similarity) within sex groups in spraints from the Tamar sanctuary

Data type	Male	Female
	Average % similarity	
absolute content (log_{10} transformed)	88.03	90.29
presence or absence	83.05	82.51
	Spraint chemical (average % contribution to similarity)	
absolute content (log_{10} transformed)	indole (1.85)	phenol (1.68)
presence or absence	phenol, 4,4'-(1-methylethylidene)bis- (2.04)	2(3H)-furanone, dihydro-5-octyl (1.92)

Table 11. Average percentage dissimilarity (plus spraint chemicals with the largest contribution to dissimilarity) between sex groups in spraints from the Tamar sanctuary

Data type	Male v Female	
	Average % dissimilarity	*Spraint chemical (average % contribution to dissimilarity)*
absolute content (log_{10} transformed)	14.61	1-nonanal (3.65)
presence or absence	22.58	2-decanol (3.63)

Comparison of spraints from Bowes sanctuary European otter individuals using Multi-Dimensional Scaling (MDS)

Absolute content data, log_{10} transformed 1 way ANOSIM [R=0.64, p=0.036]

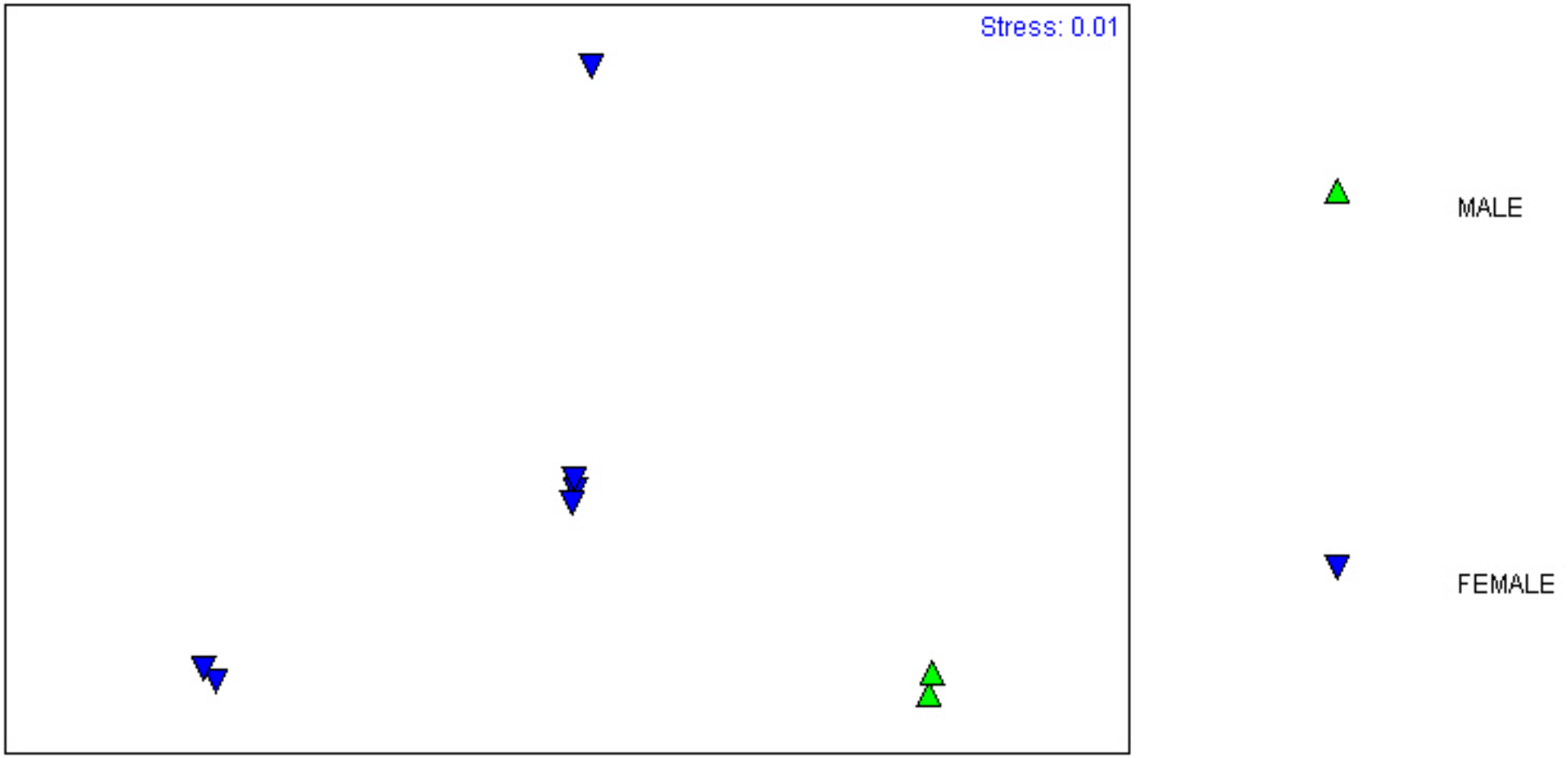


Figure 14. Multi Dimensional Scaling diagram showing relatedness between Bowes European otter sexes, based on log_{10} transformed absolute scent mark odour chemical data. 1 way ANOSIM R values indicate significant differences between the sexes.

Presence or absence data 1 way ANOSIM [R=0.80, p=0.036]

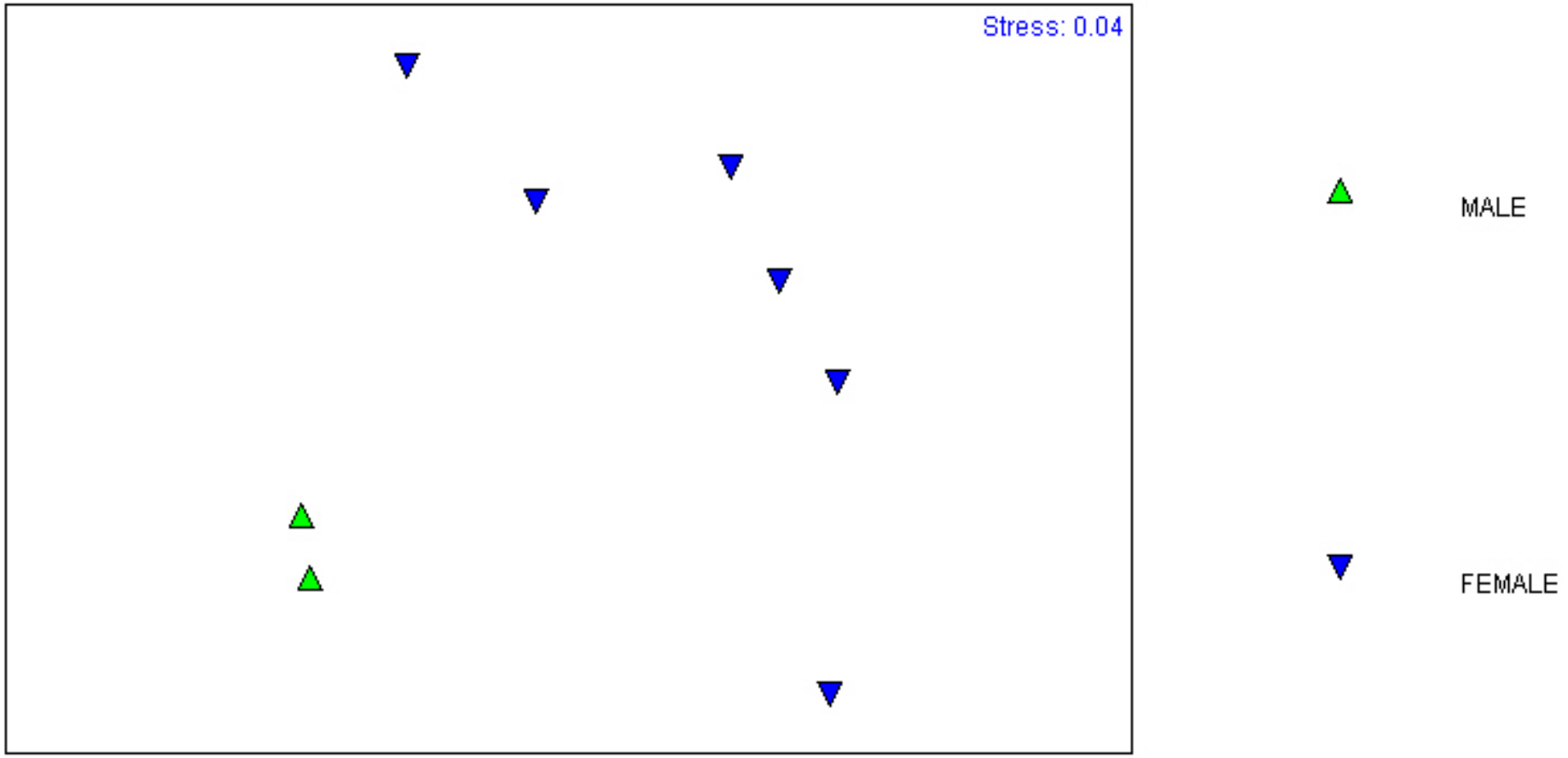


Figure 15. Multi Dimensional Scaling diagram showing relatedness between Bowes European otter sexes, based on presence or absence scent mark odour chemical data. 1 way ANOSIM R values indicate significant differences between the sexes.

Both MDS and ANOSIM analyses using spraint odour chemical data (absolute and presence or absence) showed a similar result; a clear separation of Bowes sanctuary sex groups. ANOSIM R values indicate significant differences between the sexes for both data types.

Further analysis was now carried out in order to ascertain which chemicals might be most responsible for the separation seen. Table 12 summarises the % similarity within the sex groups, plus the chemicals most responsible for these similarities. Table 13 summarises and the dissimilarity between the sex groups for each data type plus the chemicals most responsible for these dissimilarities.

SIMPER analysis

Similarity / dissimilarity summary tables

Table 12. Average percentage similarity (plus spraint chemicals with the largest contribution to similarity) within sex groups in spraints from the Bowes sanctuary

Data type	Male	Female
	Average % similarity	
absolute content (log_{10} transformed)	94.19	84.34
presence or absence	93.26	83.28
	Spraint chemical (average % contribution to similarity)	
absolute content (log_{10} transformed)	1-tert-butoxy-2-methoxy ethene (1.39)	1-tert-butoxy-2-methoxy ethene (1.91)
presence or absence	2 propenoic acid, 3-(4-methoxypheny)-2-ethyl hexyl ester (1.2)	phenol,4-4-(4-methyl ethylidene)bis (1.79)

Table 13. Average percentage dissimilarity (plus spraint chemicals with the largest contribution to dissimilarity) between sex groups in spraints from the Bowes sanctuary

Data type	Male v Female	
	Average % dissimilarity	*Spraint chemical (average % contribution to dissimilarity)*
absolute content (log_{10} transformed)	19.04	3,4,4-trimethyl-2-cyclohexen-1-one (2.82)
presence or absence	23.95	alkane-36.955 (2.68)

Comparison of European otter male & female spraints from Tamar and Bowes sanctuaries using Multi-Dimensional Scaling (MDS)

Absolute content data, log_{10} transformed Combined 2 way ANOSIM [R=0.71, p=0.001]

Figure 16. Multi Dimensional Scaling diagram showing relatedness between Tamar and Bowes European otter sexes, based on log_{10} transformed absolute scent mark odour chemical data. 2 way ANOSIM R values indicate significant differences between the sexes.

Presence or absence data Combined 2 way ANOSIM [R=0.23, p=0.107]

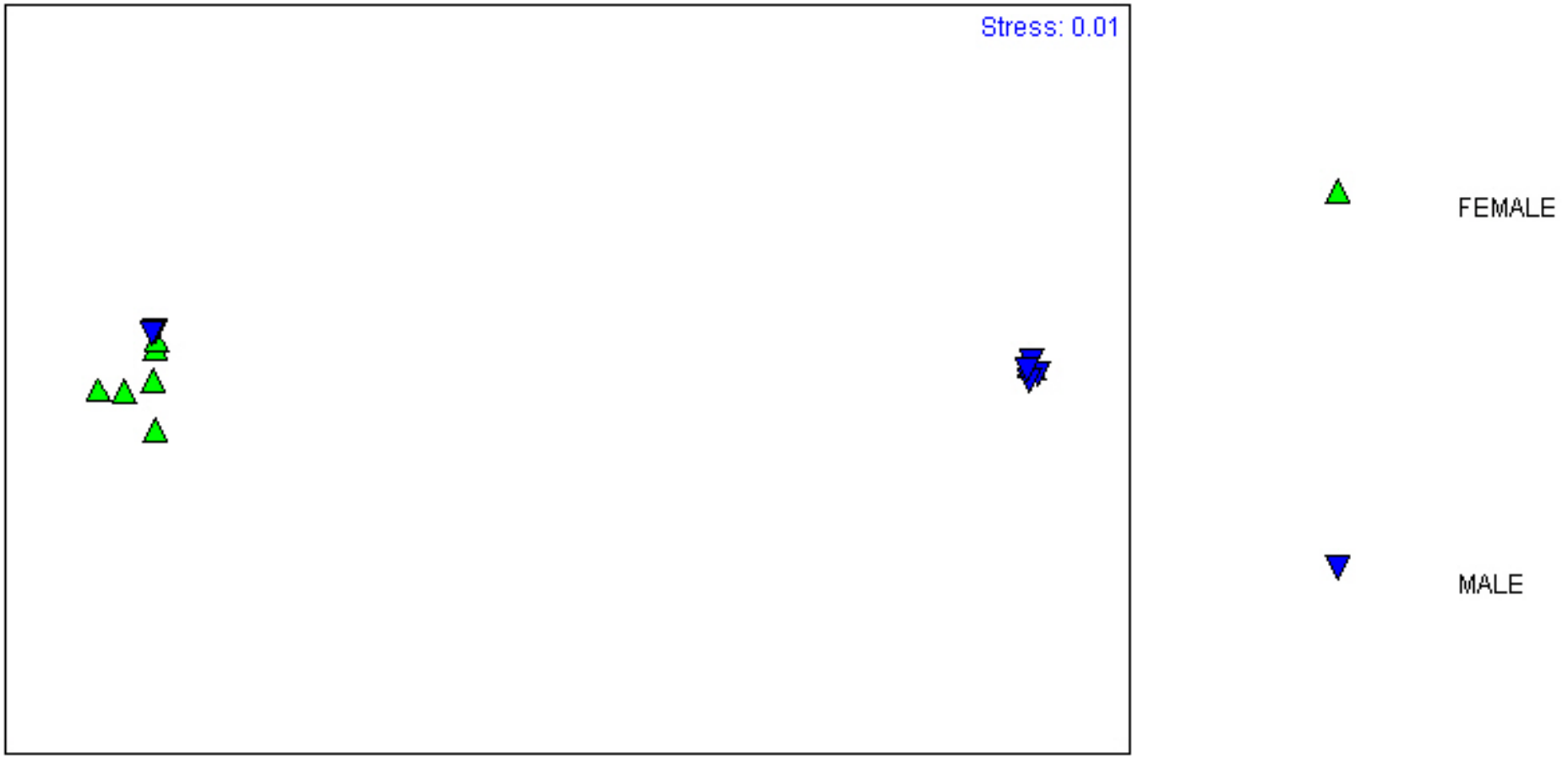


Figure 17. Multi Dimensional Scaling diagram showing relatedness between Tamar and Bowes European otter sexes, based on presence or absence scent mark odour chemical data. 2 way ANOSIM R values indicate significant differences between the sexes.

Both MDS analyses using spraint odour chemical data (absolute and presence or absence) showed a similar result; a certain level of separation between the sexes was observed within two clusters, each representing a different sanctuary. There was therefore no overall separation of male and female samples. A combined 2 way ANOSIM was used to test for differences between sex groups across all site groups. This test highlights differences between sexes whilst taking into account the fact that there are two site groups. The ANOSIM results indicate that despite the apparent division within sex groups in the MDS, a significant difference between sexes was also present. As discussed below (and shown in Figures 18 and 19) the two main clusters of samples in the MDS relate to differences in site groups but with further differences within each site group relating to sex. The 2 way ANOSIM takes into account the site groupings and indicates significant differences between sexes with the absolute data giving much stronger differences than the presence or absence data.

Further analysis was now carried out in order to ascertain which chemicals might be most responsible for the separation seen. Table 14 summarises the % similarity within the sex groups, plus the chemicals most responsible for these similarities. Table 15 summarises and the dissimilarity between the sex groups for each data type plus the chemicals most responsible for these dissimilarities.

SIMPER analysis

Similarity / dissimilarity summary tables

Table 14. Average percentage similarity (plus spraint chemicals with the largest contribution to similarity) within sex groups in spraints from the Tamar and Bowes sanctuaries combined.

Data type	Male	Female
	Average % Similarity	
absolute content (log_{10} transformed)	73.09	72.39
presence or absence	81.43	79.49
	Spraint chemical (average % contribution to similarity)	
absolute content (log_{10} transformed)	benzaldehyde (1.9)	benzaldehyde (1.9)
presence or absence	phenol, 4,4'-(1-methylethylidene) bis- (1.22)	phenol, 4,4'-(1-methylethylidene) bis- (1.29)

Table 15. Average percentage dissimilarity (plus spraint chemicals with the largest contribution to dissimilarity) between sex groups in spraints from the Tamar and Bowes sanctuaries combined.

Data type	Male v Female	
	Average % dissimilarity	*Spraint chemical (average % contribution to dissimilarity)*
absolute content (log_{10} transformed)	28.53	mexiletine (1.47)
presence or absence	19.58	2-propenoic acid, 3-(4-methoxyphenyl_-2-ethylhexyl ester (1.39)

Comparison of spraints from different sanctuaries (Tamar and Bowes) using Multi-Dimensional Scaling (MDS)

Absolute content data, log_{10} transformed Combined 2 way ANOSIM [R=1, p=0.001]

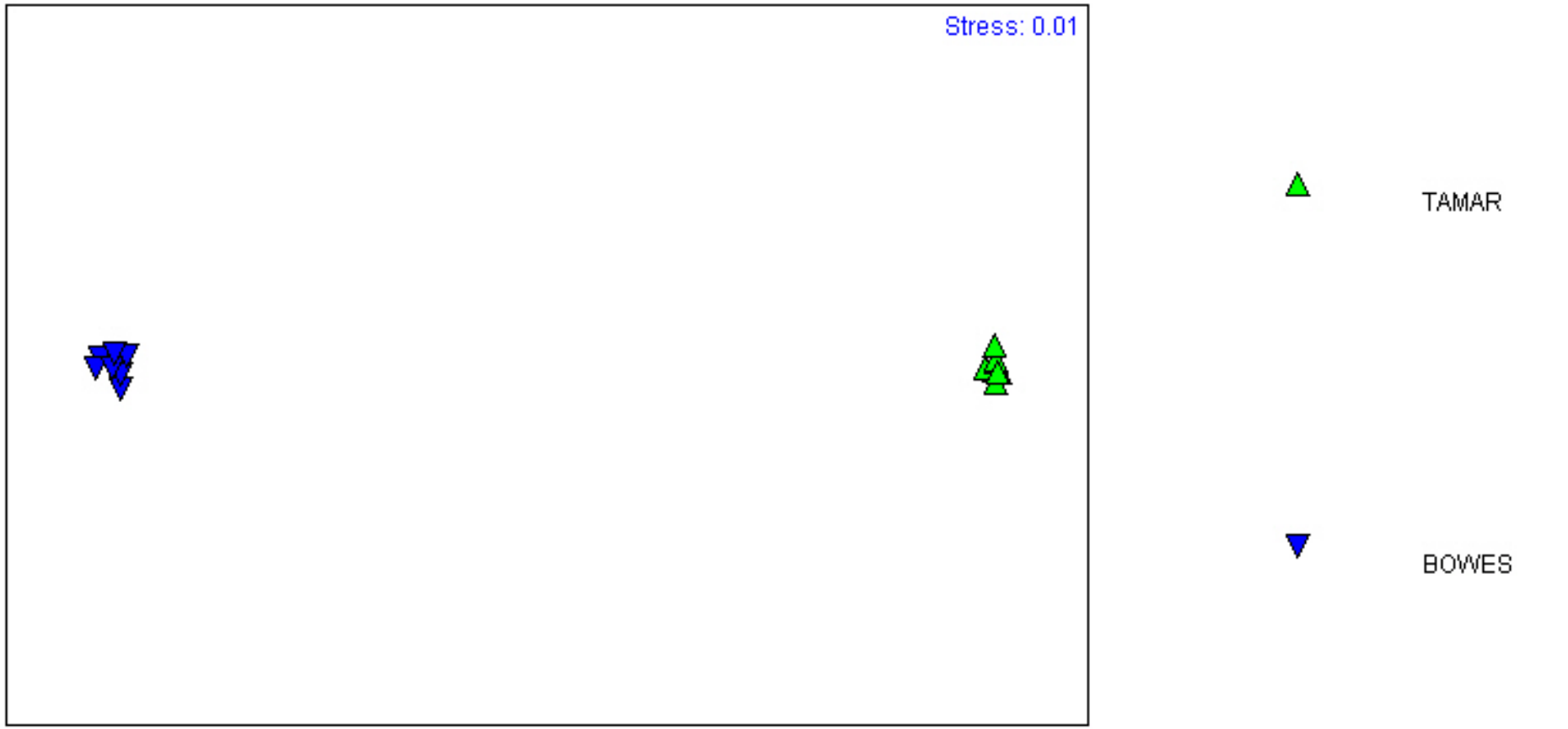


Figure 18. Multi Dimensional Scaling diagram showing relatedness between Tamar and Bowes European otter sanctuaries, based on log_{10} transformed absolute scent mark odour chemical data. 2 way ANOSIM R values indicate significant differences between the site groups.

Presence or absence data Combined 2 way ANOSIM [R=1, p=0.001]

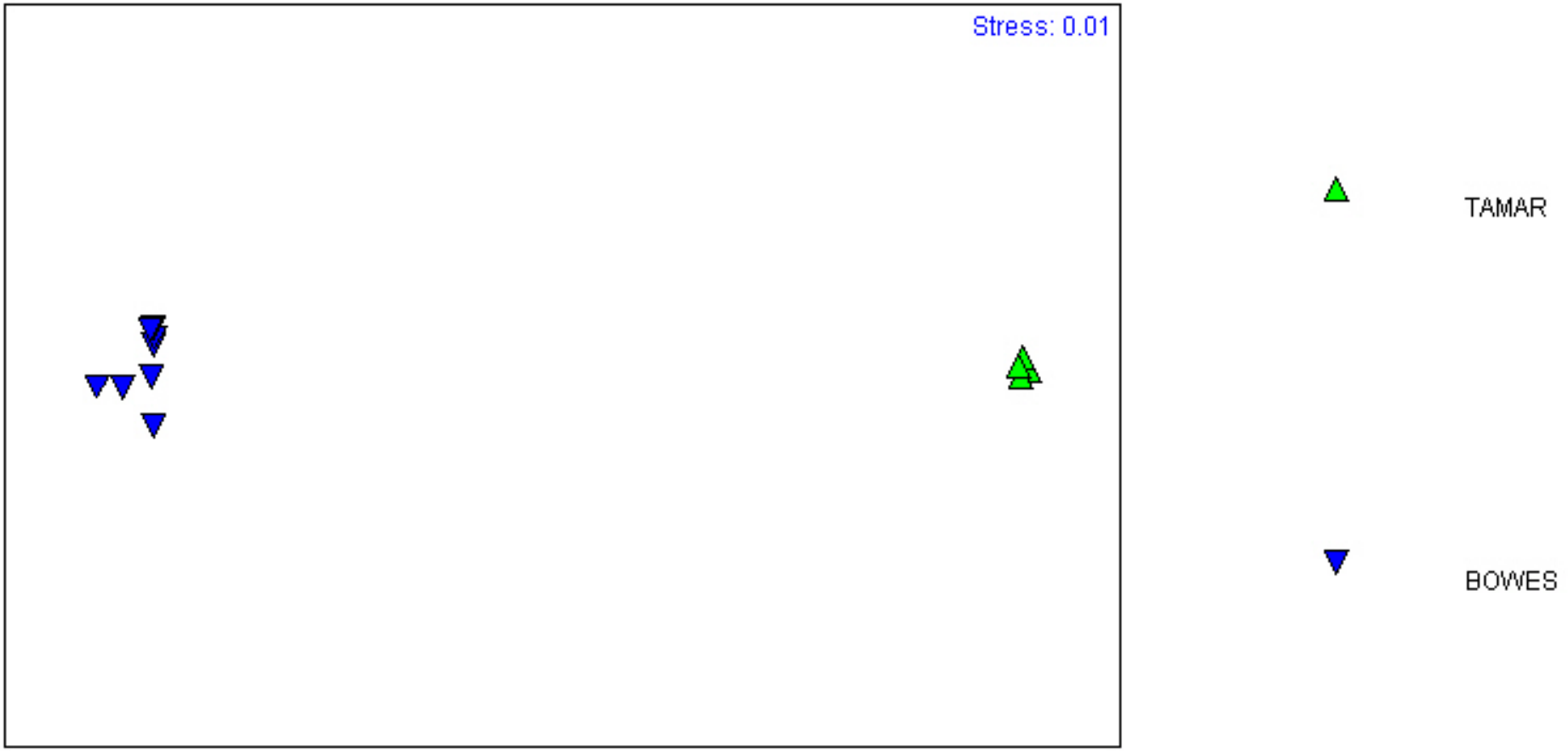


Figure 19. Multi Dimensional Scaling diagram showing relatedness between Tamar and Bowes European otter sanctuaries, based on presence or absence scent mark odour chemical data. 2 way ANOSIM R values indicate significant differences between the site groups.

Both MDS analyses using spraint odour chemical data (absolute and presence or absence) showed a similar result; a definite separation between samples from different sanctuaries was observed. A combined 2 way ANOSIM was used to test for differences between site groups across all sex groups. These results highlight the large difference in spraint odour chemical profile between site groups (R=1; p=0.001).

Further analysis was now carried out in order to ascertain which chemicals might be most responsible for the separation seen. Table 16 summarises the % similarity within the sanctuary groups, plus the chemicals most responsible for these similarities. Table 17 summarises and the dissimilarity between the sanctuary groups for each data type plus the chemicals most responsible for these dissimilarities.

SIMPER analysis

Similarity / dissimilarity summary tables

Table 16. Average percentage similarity within sanctuary groups (plus spraint chemicals with the largest contribution to similarity)

Data type	Tamar	Bowes
	Average % similarity	
absolute content (log_{10} transformed)	87.01	83.25
presence or absence	94.75	87.95
	Spraint chemical (average % contribution to similarity)	
absolute content (log_{10} transformed)	(E)-5(pentyloxy)-2-pentene (1.77)	1-tert-butoxy-2-methoxy ethene (1.84)
presence or absence	phenol, 4,4'-(1-methylethylidene) bis- (1.08)	pentatriacontane (1.14)

Table 17. Average percentage dissimilarity between sanctuary groups (plus spraint chemicals with the largest contribution to dissimilarity)

Data type	Tamar v Bowes	
	Average % dissimilarity	*Spraint chemical (average % contribution to dissimilarity)*
absolute content data, log_{10} transformed	39.45	1-tert-butoxy-2-methoxy ethene (1.96)
presence or absence data	29.56	2-propenoic acid, 3-(4-methoxyphenyl-2-ethylhexyl ester (1.71)

Comparison of spraints from Chestnut sanctuary European otter individuals using Multi-Dimensional Scaling (MDS)

Absolute content data, log_{10} transformed 1 way ANOSIM [R=0.02, p=0.359]

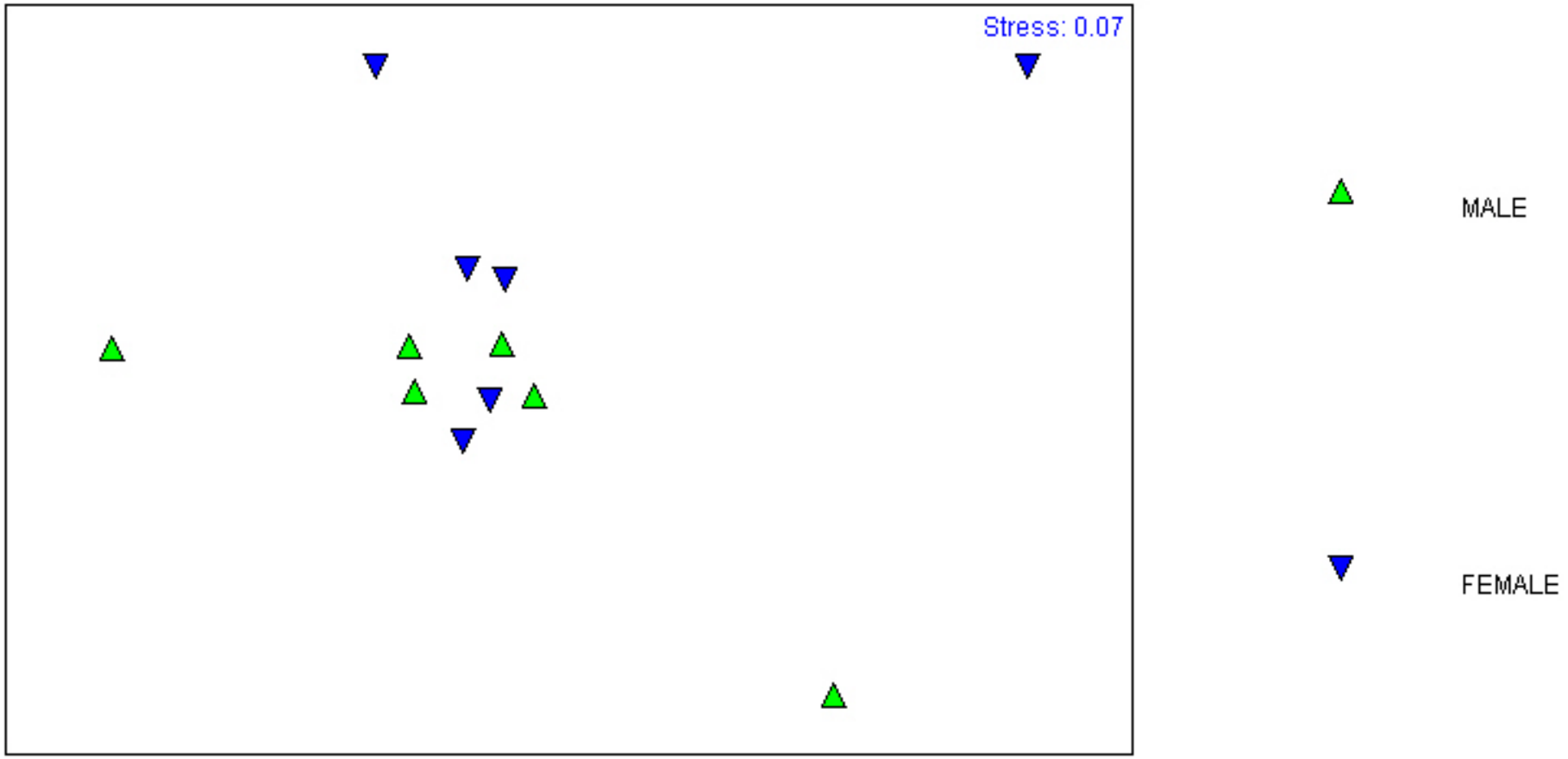


Figure 20. Multi Dimensional Scaling diagram showing relatedness between Chestnut European otter sexes, based on log_{10} transformed absolute scent mark odour chemical data. 1 way ANOSIM R values indicate no significant differences between the sexes.

Presence or absence data 1 way ANOSIM [R=-0.04, p=0.673]

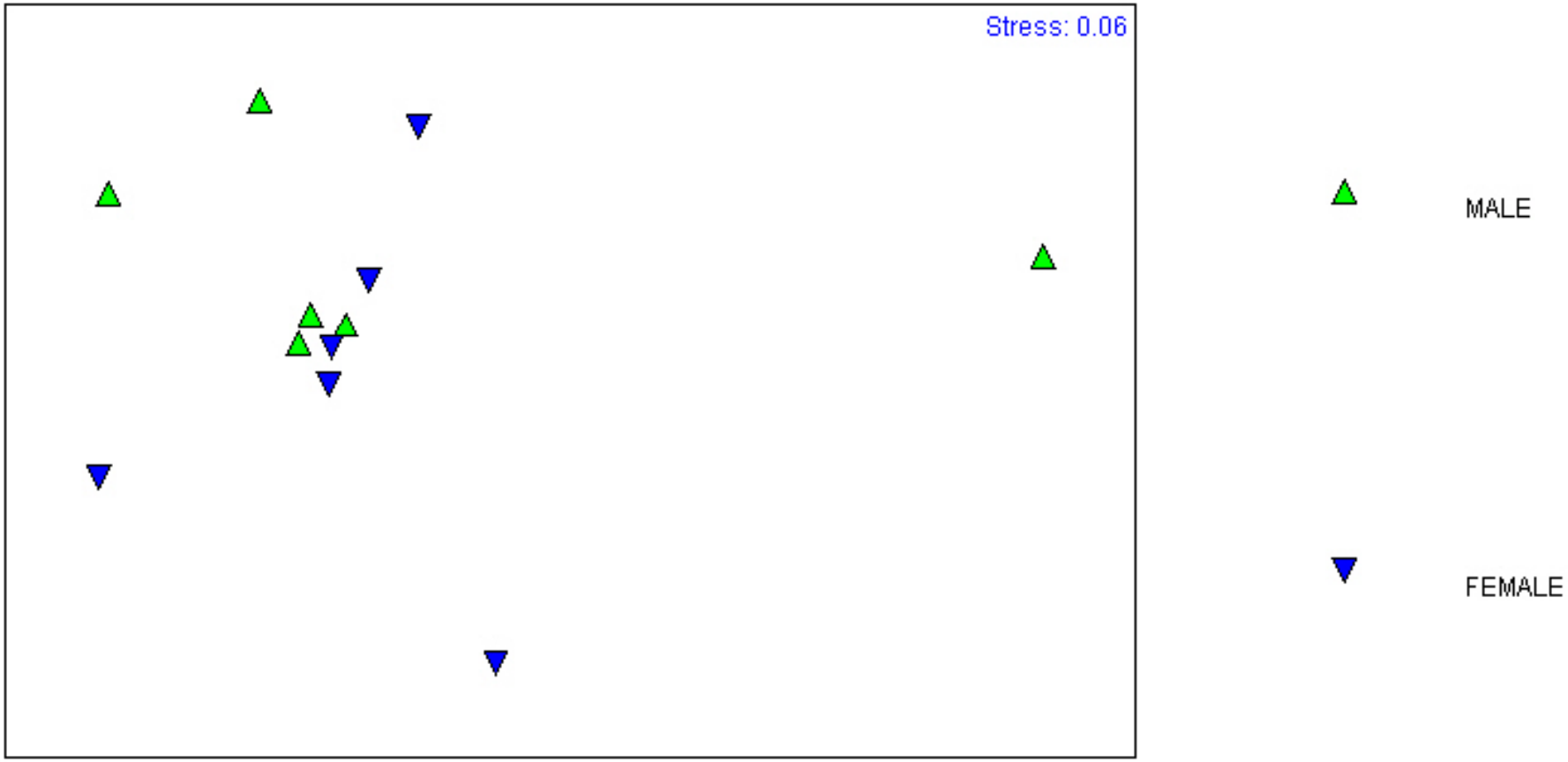


Figure 21. Multi Dimensional Scaling diagram showing relatedness between Chestnut European otter sexes, based on presence or absence scent mark odour chemical data. 1 way ANOSIM R values indicate no significant differences between the sexes.

Both MDS analyses using spraint odour chemical data (absolute and presence or absence) showed a similar result; a possible clustering of male samples within a wider range of female samples. The ANOSIM analyses using spraint odour chemical data (absolute and presence or absence) indicated no clear separation of Chestnut sanctuary sex groups. ANOSIM R values indicate no significant differences between the sexes for both data types.

Further analysis was now carried out in order to ascertain which chemicals might be most responsible for the separation seen. Table 18 summarises the % similarity within the sex groups, plus the chemicals most responsible for these similarities. Table 19 summarises and the dissimilarity between the sex groups for each data type plus the chemicals most responsible for these dissimilarities.

SIMPER analysis

Similarity / dissimilarity summary tables

Table 18. Average percentage similarity (plus spraint chemicals with the largest contribution to similarity) within sex groups in spraints from the Chestnut sanctuary

Data type	Male	Female
	Average % similarity	
absolute content (log_{10} transformed)	76.22	73.75
presence or absence	70.07	71.11
	Spraint chemical (average % contribution to similarity)	
absolute content (log_{10} transformed)	C_6, hexane (11.3)	C_6, hexane (10.32)
presence or absence	C_6, hexane (12.14)	cyclobutanol (11.06)

Table 19. Average percentage dissimilarity (plus spraint chemicals with the largest contribution to dissimilarity) between sex groups in spraints from the Chestnut sanctuary

Data type	Male v Female	
	Average % dissimilarity	*Spraint chemical (average % contribution to dissimilarity)*
absolute content data, log_{10} transformed	25.79	isopropyl alcohol (9.00)
presence or absence data	29.10	isopropyl alcohol (8.57)

DISCUSSION

Bowes and Tamar Sanctuaries

Overall, 143 peaks were seen, but there was variation between individuals with the number of peaks per animal ranging from 87 - 113.

For the Tamar Sanctuary alone there were a total of 104 peaks, and the variation in the number of peaks per animal ranged from 90 - 104

For the Bowes Sanctuary alone there were a total of 121 peaks, and the variation in the number of peaks per animal ranged from 87 – 113.

Categories of compound extracted from captive European otter spraint material included: alkanes/alkenes; aldehydes; ketones; alcohols; carboxylic acids; benzene compounds; nitrogen compounds; sulphur compounds; and others (see Tables 3 and 4).

Alkanes/alkenes

Overall 44 alkanes/alkenes were seen, 32 in the Tamar European otter spraints and 36 in the Bowes European otter spraints. Examples included 6-methyl-1-heptene, 3-carene, C9, C10, C11, C13, 6-ethyl-2-methyl-decane, 2,3,5-trimethyl-decane, and 1-heptadecene). No alkanes/alkenes were identified in previous work on otter anal sac secretion (Bradshaw et al, 2001).

The anal sac secretion of the wolverine, *Gulo gulo* has been shown to contain a variety of alkanes/alkenes: nonane, a-pinene, b-pinene, 3-methylnonane, decane, and undecane (Wood et al, 2005).

Aldehydes

Overall 22 aldehydes were seen, 19 in the Tamar European otter spraints and 22 in the Bowes European otter spraints. This included straight chain aldehydes (C_7-C_{12}) plus benzaldehyde. Aldehydes also featured prominently in work carried out on otter anal gland secretion (Bradshaw et al, 2001). See Table 20.

Table 20: aldehydes found in anal sac secretion of the European otter using purge + trap and GC-MS (Bradshaw et al, 2001)

2-methyl-propanal	hexanal
3-methyl-propanal	5-methyl-hexanal
2-methyl-butanal	benzaldehyde

Brinck et al (1978) showed the presence of aldehydes in mink, *Mustela vison* anal sac secretion using the infra red spectrum. Benzaldehyde has been identified in pine marten, *Martes martes* anal sac secretion (Brinck et al, 1983) and the anal sac secretion of the wolverine which also contained 4-hydroxybenzaldehyde (Wood et al, 2005).

Ketones

Overall 21 ketones were seen, 15 in the Tamar European otter spraints and 19 in the Bowes European otter spraints. Examples included 2-tridecanone, 6-methyl-5-hepten-2-one, 4-undecanone, 2-tridecanone, 9-decen-2-one). No ketones were identified in previous work on European otter anal sac secretion (Bradshaw et al, 2001).

Other ketones found in the anal sac secretion of mustelid species include *o*-aminoacetophenone in the Siberian weasel (*Mustela sibirica fortanieri*) and the steppe polecat (*Mustela eversmann admirati*) (Zhang et al, 2003) and 3-octanone in wolverine anal sac secretion (Wood et al, 2005).

Alcohols and phenols

Overall 16 alcohols were seen, 12 in the Tamar European otter spraints and 13 in the Bowes European otter spraints (see Tables 4 and 5). Examples included heptanol, 1-hepten-3-ol, phenol, benzyl alcohol, 2-decanol). 1-pentanol was identified in previous work on *European otter* anal sac secretion (Bradshaw et al, 2001).

The anal sac secretion of the wolverine has been shown to contain a variety of alcoholic compounds: 3-methyl-1-butanol, 1-octen-3-ol, benzyl alcohol, linalool, 2,3-dimethylcyclohexanol, 2-phenylethanol, 1,2-hexadecanediol, a-tocopherol, and cholesterol (Wood et al, 2005).

Carboxylic acids

Overall 12 carboxylic acids and carboxylic acid esters were seen, 7 in the Tamar European otter spraints and 10 in the Bowes European otter spraints (see Tables 4 and 5). Previous work on European otter anal sac secretion (Bradshaw et al, 2001) identified the presence of 2 carboxylic acid ethyl-esters (2-methyl-butanoic acid ethyl-ester and 2-methyl-butanoic acid ethyl-ester).

Short-chain carboxylic acids were found in the anal sac secretion of both the pine marten and beech marten, *Martes foina* (Schildknecht & Birkner, 1983). The anal sac secretion of the wolverine also contained a large number of carboxylic acids (See Table 21)

Table 21: carboxylic acids found in anal sac secretion of the wolverine using solvent extraction and GC-MS (Wood et al, 2005)

2-methylpropanoic acid	phenylacetic acid
3-methylbutanoic acid	nonanoic acid
2-methylbutanoic acid	3-phenylpropanoic acid
pentanoic acid	decanoic acid
2-methylpentanoic acid	2-methyldecanoic acid
hexanoic acid	2-decenoic acid
2-methylhexanoic acid	dodecanoic acid
heptanoic acid	tetradecanoic acid
octanoic acid	pentadecanoic acid
2-octenoic acid	(Z)-9-hexadecenoic acid
7-octenoic acid	hexadecanoic acid
2-methyloctanoic acid	linoleic acid
4-methyloctanoic acid	oleic acid
	stearic acid

Some of the wolverine match or closely match those found in the otter spraint material.

All those compounds identified in the anal sac secretion of the European Badger, *Meles meles* are high molecular weight fatty acids of low volatility in the range C_{14}-C_{24} (Davies et al 1988).

Benzene compounds

Overall 5 benzene compounds were seen, 4 in the Tamar European otter spraints (1-methylethyl)-benzene, 1,4-dichlorobenzene, diethyl benzene, and another form of benzene) and 1 in the Bowes European otter spraints (1(1-ethylnonyl)-benzene) (see

Tables 4 and 5). No benzene compounds were seen in the previous work on *European otter* anal sac secretion (Bradshaw et al, 2001).

The anal sac secretion of the wolverine has been shown to contain 4-hydroxybenzaldehyde (Wood et al, 2005).

Nitrogen compounds

Overall 5 nitrogen compounds were seen, 3 in the Tamar European otter spraints and 5 in the Bowes European otter spraints (see Table 4 and 5). No nitrogen compounds were seen in the previous work on European otter anal sac secretion (Bradshaw et al, 2001).

Indole has been found in the anal sac secretion of the mink (Brinck et al, 1978; Schildknecht et al, 1976 and Sokolov et al, 1980); the stoat, (Crump, 1980b: Brinck et al, 1983); the ferret (Crump, 1980a); the steppe polecat and the Siberian weasel (Zhang et al, 2003); the weasel, *Mustela nivalis* (Brinck et al, 1983); and the wolverine (Wood et al, 2005). It has also been confirmed in the anal sac secretion of a number of other carnivores (Albone & Grönneberg, 1977; Apps et al, 1989; Hagey & MacDonald, 2003).

Indole was found in all European otter spraint samples from the Tamar and Bowes sanctuaries. Although it cannot be confirmed (no SPME analysis of anal sac secretion alone was carried out) it is likely that the indole found here was a faecal constituent. Indole is formed in the stomach from deamination and decarboxylation of the amino acid, tryptophan (Deslandes et al, 2001). To date, no other work has confirmed indole as being present in European otter, spraints or anal sac secretion. For example, the most recent work on European otter, using GC/MS analysis of anal sac secretion did not identify indole as a constituent (Bradshaw et al, 2001) and this may support a faecal origin for indole in spraints.

Other nitrogen-containing compounds found in the anal sac secretion of mustelid species include *o*-aminoacetophenone in the Siberian weasel and the steppe polecat (Zhang et al, 2003) and quinoline in the ferret (Crump, 1980a).

Sulphur compounds

Overall 2 sulphur compounds were seen, 2 in the Tamar European otter spraints (dimethyl trisulfide, 1,1'-sulfonylbis 4-chloro-benzene) and 1 in the Bowes European otter spraints (1,1'-sulfonylbis 4-chloro-benzene) (see Tables 4 and 5). Many mustelid anal sac secretions have been shown to contain sulphur compounds. These include those of the stoat (Crump 1980a); the ferret (Crump 1980b); the mink (Sokolov et al, 1980); the steppe polecat (Zhang et al, 2002) and the weasel (Buglass et al, 1991). However, sulphur compounds were not found in the anal sac secretion of the European badger, (Davies et al, 1988). Many sulphur compounds are well known microbial products (Sokolov et al, 1980).

In this study, dimethyl trisulphide was found only in the spraints of those European otter from the Bowes sanctuary. If this compound has a dietary origin, then differences in otter diet between sanctuaries might account for its presence and absence. However, dimethyl trisulphide (plus dimethyl-disulphide) has been previously identified in the anal sac secretion of wild European otter (Bradshaw et al, 2001).

Otter spraint material contained a very small proportion of sulphur compounds in comparison with the anal sac secretion compounds of the *Mustela* species. Compounds found in the *Mustela* are similar with most (in particular, the sulphur-containing compounds) being specific to the genus (Zhang et al, 2003). *Mustela* sulphur-containing compounds are typically thietanes, dithiolanes and dithiacyclo-compounds. None of these compound types are present in otter spraint material.

In Antarctic Procellariiform seabirds, dimethyl sulphide acts as a foraging cue (Nevitt et al, 1995). A related compound, dimethyl disulphide has been identified in the anal sac secretion of the mink (Sokolov et al, 1980). It has also been identified in Eurasian otter anal sac secretion (Bradshaw et al, 2001). Dimethyl disulphide is an important olfactory compound known to have attractant properties, e.g., it has been linked with sexual attraction in the hamster (O'Connell et al, 1979; Petrulis & Johnston, 1995).

Others

Overall 15 other compounds were seen, 10 in the Tamar European otter spraints and 14 in the Bowes European otter spraints (see Tables 3 and 4). Examples include

furan-2-propyl, xylofuranose, a-cyclocitral, nimorazole, (E,E)-farnesol, bis(2-methoxyethyl)phthalate, bis(2-ethylhexyl)phthalate. Work on otter anal sac secretion (Bradshaw et al, 2001) revealed the presence of 2-ethyl-furan and 2-pentyl-furan and a study on the anal gland secretion of the wolverine (Wood et al, 2005) also identified these types of compounds: 3-methyl-dihydro-2(3H)furanone, 4-methyl-dihydro-2(3H)furanone, 2-pentylfuran, and 5,6,7,7-tetrahydro-2(4H)benzofuranone.

It is clear when comparing the types of compound found in European otter spraints to those found in the anal sac secretion of other mustelid species, that there are tremendous similarities.

Comparisons of sanctuaries, sexes and individuals

Overlap in compound composition within and between the sex groups in captive otter spraints from the Tamar otter sanctuary alone

In terms of compound overlap, females overlapped other females significantly more in compound composition than they did males. A similar significant difference was not found for the males; in fact males overlapped females significantly more in compound composition than they did other males (see Figure 7). Therefore, it is still unclear what role, if any, the overall compound composition (in terms of presence or absence) plays in the identification of sex within the Tamar sample group.

No clearcut male/female difference is seen – females appear to have a distinctive spraint compound make-up, however males are less distinctive. Examining the raw data reveals very similar profiles between males and females, with very few digital coding differences. Pentatriacontane, for example, occurs in 3 of the 4 male samples, but in none of the female samples. Conversely, 2-methyl-2-propenoic acid *(8.269)* occurs in all the female samples, but none of the males (see Table 22).

Table 22. Male and female compounds in Tamar sanctuary spraints.

in males, but not females	**in females, but not males**
pentatriacontane	2-methyl-2-propenoic acid *(8.269)* 4,8-dimethyl 1-nonanol *(8.386)*

NB – neither 2-methyl-2-propenoic acid or 4,8-dimethyl 1-nonanol appeared in the spraint samples of otters from the Bowes sanctuary. Pentatriacontane appeared in all samples (male and female).

Overlap in compound composition within and between the sex groups in captive otter spraints from the Bowes otter sanctuary alone

In terms of compound overlap, males overlapped other males significantly more in compound composition than they did females (NB this could be explained by the low number of male samples available for this part of the study). A similar significant difference was not found for the females; in fact females overlapped males significantly more in compound composition than they did other females (see Figure 8). Therefore, it is still unclear what role, if any, the overall compound composition (in terms of presence or absence) plays in the identification of sex within the Bowes sample group.

No clearcut male/female difference is seen – males appear to have a distinctive spraint compound make-up, however females are less distinctive. Examining the raw data reveals very similar profiles between males and females, with very few digital coding differences (See Table 23).

Table 23. Male and female compounds in Bowes sanctuary spraints.

in males, but not females	**in females, but not males**
3,4,4-trimethyl-2cyclohexen-1- one *(9.479)*	C_{10} *(6.127)*
cyclohexaneethanol, acetate *(14.147)*	3-heptanone, 2,4-dimethyl- *(7.328)*
9-octadecenal, (Z)- *(29.152)*	propyl-thiopane *(8.562)*
eicosanoic acid *(37.705)*	

NB – propyl-thiopane, 3,4,4-trimethyl-2cyclohexen-1- one, cyclohexaneethanol, acetate and 9-octadecenal, (Z)- did not appear in the spraint samples of otters from the Tamar sanctuary. C_{10} and 3-heptanone, 2,4-dimethyl- appeared in all samples (male and female). Eicosanoic acid appeared in all females and only one male in trace amounts.

Overlap in compound composition within and between the sex groups in captive otter spraints from both Tamar and Bowes otter sanctuaries

In terms of compound overlap, males overlapped other males more in compound composition than they did females. A similar difference was not found for the females; they overlapped males more in compound composition than they did other females (see Figure 9). However, none of these differences were found to be statistically significant. This is a much less distinct result than those seen when the Tamar and Bowes sanctuary data was examined separately. Both sets of data included differences between males and females in terms of presence or absence of spraint compounds (digital coding) – see Tables 22 and 23. However, these differences do not involve the same chemicals for both sets of data (see notes at bottom of Tables 22 and 23 for details). This means that although there are possible male / female differences within each sanctuary, no such distinction is observed when the data is combined. Therefore, the combined (Tamar and Bowes) data for compound composition, in terms of presence or absence (digital coding, Sun & Müller-Schwarze, 1998, 1999) seems to play no role in the identification of sex. If this is the case, then any individual or sexual differences may alternatively be conveyed through variation in the absolute or relative abundance of these compounds (analogue coding, Sun & Müller-Schwarze, 1998, 1999), although, of course there may be an element of both. This possibility was explored using Multi- Dimensional Scaling (these results are examined later in this discussion).

Overlap in compound composition within and between the different sanctuary groups (Tamar and Bowes) in captive otter spraints

In terms of compound overlap, Bowes spraints overlapped other Bowes spraints significantly more in compound composition than they did Tamar spraints. A similar significant difference was found for the Tamar spraints; with Tamar spraints overlapping other Tamar spraints significantly more in compound composition than they did Bowes spraints (see Figure 10).

Therefore, in terms of presence or absence of compounds in the headspace, the spraints of otters from different sanctuaries are distinguishable.

Upon examination of the raw data it can easily be seen that there are both digital and analogue coding differences between the two sample sets.

Digital

For example, both 2-decanol (*13.17*) and 17-pentatriacontene (*16.688*) appear in the Tamar spraints, but not the Bowes spraints. Conversely, cyclopentanol, 2-methyl-, trans- *(2.21)* and heptanal *(3.793)* appear in the Bowes spraints, but not the Tamar spraints.

Analogue

For example, (E)-5(pentyloxy)-2-pentene *(4.684)* appears as a substantial peak in the Tamar spraints, but only in trace amounts in the Bowes spraints. Conversely, 2-tridecanone *(3.543)* appears as a substantial peak in the Bowes spraints, but only in trace amounts in the Tamar spraints.

Overlap in compound composition within and between the sex groups in captive otter spraints from the Chestnut otter sanctuary alone

In terms of compound overlap, females overlapped other females more in compound composition than they did males. A similar difference was not found for the males; in fact males overlapped females more in compound composition than they did other males (Figure 11). None of the differences were statistically significant. The lack of differentiation between males and females in the Chestnut sanctuary in terms of compound overlap can be explained by the absence of any male or female specific compounds in the data. Individual or sexual differences may alternatively be conveyed through variation in the relative abundance of these compounds (analogue coding, Sun & Müller-Schwarze, 1998, 1999), although, of course there may be an element of both. This possibility was explored using Multi- Dimensional Scaling (these results are examined in the following sections).

The analysis of the Chestnut sanctuary data was carried out with a different SPME fibre than that used for the other sanctuaries (Tamar and Bowes). See Table 24.

Table 24. Fibre types used

Spraint sample set	Fibre type used
Bowes + Tamar	65µm polydimethylsiloxane/divinylbenzene (PDMS/DVB) -used for volatiles, amines and nitroaromatic compounds.
Chestnut	100µm polydimethylsiloxane (PDMS) -used for low molecular weight / volatile compounds

The 100µm PDMS fibre is more suited to extracting low molecular weight / volatile compounds and this was reflected in the data produced. Only 19 compounds were detected compared to 104 for the Tamar sanctuary and 120 for the Bowes sanctuary. Most of the 19 compounds eluted at start of the chromatogram (see Figure 5). Data from both the Tamar and Bowes sanctuaries (when examined separately) gave an indication of a male / female split; however, this was not quite as distinct for the Chestnut sanctuary data. [NB none of the data from any sanctuary showed any real indication of clustering of samples at an individual level]. Scent marks conveying messages of individual identity or sex would need to be designed to persist in the environment long after the signalling animal has moved on (Bradbury & Vehrencamp, 1998). Therefore, this type of message would be unlikely to be found in highly

volatile compounds as it would fade relatively quickly. The presence or absence of the more volatile compounds extracted with the 100µm PDMS fibre is more likely to be an indicator of spraint age (see Chapter 3).

The absolute, percentage and presence or absence data was used to carry out comparisons using Multi- Dimensional Scaling. These analyses were based on analogue (and digital) coding rather than digital coding alone.

Comparisons using Multi – Dimensional Scaling (MDS)

Separate analysis of Tamar and Bowes sanctuary data

The Multi-Dimensional Scaling analysis for both data types indicate that the composition of the spraint chemical odour profile in male and female European otter is different (when each data set is considered separately). A certain level of separation and clustering of the sexes was seen. This appears to be a better result than that observed with the earlier overlap analysis, which was based purely on presence or absence of chemicals. Here, the absolute amounts of each chemical are also considered. All differences between the sexes (in each separate sanctuary data set) were found to be statistically significant.

Tamar sanctuary

Table 10 for the Tamar sanctuary samples shows that for absolute content data each sex group has a different dominant chemical (in terms of average similarity): male - indole; female – phenol. Presence or absence data have different dominant chemicals altogether (male - phenol, 4,4'-(1-methylethylidene)bis-; female - 2(3H)-furanone, dihydro-5-octyl). Furthermore, the SIMPER analysis indicated that there were 5 chemicals common to both males and females in the absolute content data. In contrast, none of these 5 chemicals appeared in the top ten spraint odour chemicals responsible for within-group average similarities in the presence or absence data. Table 11 shows that the dominant chemicals in terms of average percentage dissimilarity are all different for each of the data types (absolute content – 1-nonanal; presence or absence - 2-decanol). The SIMPER analysis showed that the absolute content data shared 7 dissimilarity chemicals with presence or absence data. So, although different chemicals (for both within group similarity and between groups dissimilarity) have varying levels of importance depending on the data type used, they all produce a similar result as demonstrated by their respective MDS diagrams.

Both indole and phenol, 4,4'-(1-methylethylidene) bis- appeared to be present in males and females in similar amounts. These chemicals appeared in both the male and female top ten of similarity within group chemicals. In contrast phenol appeared in equal amounts in the female samples, but was more varied in the male samples (an

example of analogue coding). Phenol only appeared in the female top ten of similarity within group chemicals.

1-nonanal appeared to be present in female samples in similar amounts, but was more varied in the male samples (an example of analogue coding). In contrast, 2-decanol appeared in the male samples, but not in the female samples (an example of digital coding).

Bowes sanctary

Table 12 for the Bowes sanctuary samples shows that for absolute content data each sex group has the same dominant chemical (in terms of average similarity) - 1-tert-butoxy-2-methoxy ethene. Presence or absence data have different dominant chemicals altogether (male – 2-propenoic acid, 3-(4-methoxypheny)-2-ethyl hexyl ester; female - phenol,4-4-(4-methyl ethylidene) bis). Furthermore, the SIMPER analysis indicated that there were 6 chemicals common to both males and females in the absolute data. In contrast, none of these 6 chemicals appeared in the top ten spraint odour chemicals responsible for within-group average similarities in the presence or absence data. Table 13 shows that the dominant chemicals in terms of average percentage dissimilarity are different for each of the data types (absolute content – 3,4,4-trimethyl-2-cyclohexen-1-one; presence or absence - alkane). The SIMPER analysis showed that the absolute data share 7 dissimilarity chemicals with presence or absence data. Again, although different chemicals (for both within group similarity and between groups dissimilarity) have varying levels of importance depending on the data type used, they all produce a similar result as demonstrated by their respective MDS diagrams.

Both 1-tert-butoxy-2-methoxy ethene (absolute content) and 2-propenoic acid, 3-(4-methoxypheny)-2-ethyl hexyl ester appeared to be present in males and females in similar amounts. In contrast 1-tert-butoxy-2-methoxy ethene appeared in equal amounts in the male samples, but was more varied in the female samples (an example of analogue coding).

3,4,4-trimethyl-2-cyclohexen-1-one ($\log_{10}$ absolute) and alkane-36.955 were both present in males, but not in females (an example of digital coding).

Combined analysis of Tamar and Bowes sanctuary data

When the Tamar and Bowes sanctuaries are considered together, the MDS analysis for all data types show the data splitting into two definite groupings on the basis of sanctuary type. However, within the sanctuary groups, the male / female split seen when the sanctuary data was examined separately remain. Combined 2 way ANOSIM was used to test for differences between:

- sex groups across all site groups (ANOSIM results indicate despite the apparent division within sex groups in the MDS, a significant difference between sexes was also present)
- site groups across all sex groups (a statistically significant difference between site groups was seen for both data types (absolute and presence or absence)

Sex groups

Table 14 for the combined sanctuary samples shows that for absolute content data each sex group had the same dominant chemical (in terms of average similarity): benzaldehyde. For presence or absence data each sex group had the same dominant chemical (phenol, 4,4'-(1-methylethylidene) bis-). The SIMPER analysis also indicated that there were 8 chemicals common to both males and females in the absolute content data. Only 1 of these 8 chemicals appeared in the top ten spraint odour chemicals responsible for within-group average similarities in the presence or absence data. Table 15 shows that the dominant chemicals in terms of average percentage dissimilarity are all different for both of the data types (absolute content – mexiletine; presence or absence - 2-propenoic acid, 3-(4-methoxyphenyl-2-ethylhexyl ester). The SIMPER analysis showed that the absolute content data shared only 1 (C_{13}) with presence or absence data. Again, although different chemicals (for both within group similarity and between groups dissimilarity) have varying levels of importance depending on the data type used, they all produce a similar result as demonstrated by their respective MDS diagrams.

Both benzaldyhyde and phenol, 4,4'-(1-methylethylidene) bis- appeared to be present in males and females in similar amounts.

Sanctuary groups

Table 16 for the combined sanctuary samples shows that for absolute content data each sanctuary group had a different dominant chemical (in terms of average similarity): Tamar - (E)-5(pentyloxy)-2-pentene; Bowes - 1-tert-butoxy-2-methoxy ethene. Presence or absence data have different dominant chemicals altogether (Tamar - phenol, 4,4'-(1-methylethylidene) bis-; Bowes - pentatriacontane). In addition, the SIMPER ananlysis indicated that there were 4 chemicals common to both males and females in the absolute data. None of these 4 chemicals appeared in the top ten spraint odour chemicals responsible for within-group average similarities in the presence or absence data. Table 17 shows that the dominant chemicals in terms of average percentage dissimilarity are different for absolute content (1-tert-butoxy-2-methoxy ethane) and for the presence or absence data type (2-propenoic acid, 3-(4-methoxyphenyl-2-ethylhexyl ester). The SIMPER analysis showed that the absolute content data shared only 1 dissimilarity chemical (C_{13}) with presence or absence data. Although different chemicals (for both within group similarity and between group dissimilarity) have varying levels of importance depending on the data type used, they all produce a similar result as demonstrated by their respective MDS diagrams. This may indicate that a number of mechanisms for discrimination of the sanctuaries are operating simultaneously.

(E)-5(pentyloxy)-2-pentene was present in Tamar and Bowes sanctuary otters, but in larger amounts in Tamar otters and smaller amounts in Bowes otters (analogue coding). 1-tert-butoxy-2-methoxyethane was present in Bowes sanctuary otters, but not in Tamar sanctuary otters (digital coding). Lastly, phenol, 4,4'-(1-methylethylidene) bis- was present in both Tamar and Bowes sanctuary otters in equal amounts.

1-tert-butoxy-2-methoxyethane and 3-(4-methoxyphenyl_-2-ethylhexyl ester contributed to the dissimilarity between sanctuaries using an identical coding mechanism. They are present in the Bowes sanctuary otters, but not the Tamar sanctuary otters (digital coding).

Analysis of Chestnut sanctuary data

The MDS analyses for all data types indicate a possible clustering of male samples within a wider range of female samples. This appears to be a better result than that observed with the earlier overlap analysis, which was based purely on presence or absence of chemicals. Here, the absolute content amounts of each chemical are also considered. No significant difference was seen between the sexes for the Chestnut sanctuary data.

Table 18 for the Chestnut sanctuary samples shows that for absolute content data, each sex group has the same dominant chemical (in terms of average similarity): C_6, hexane. Presence or absence data had a slightly different result (male - C_6, hexane; female - cyclobutanol). In addition, the SIMPER analysis indicated that there were 6 chemicals common to both males and females in the absolute content data and these were common to males and females in the presence or absence data. Table 19 shows the dominant chemical in terms of average percentage dissimilarity for both absolute and presence or absence data is isopropyl alcohol. 8 dissimilarity chemicals in total are common to both absolute content data and presence or absence data. Therefore, in contrast to the data from the other two sanctuaries, all types of the Chestnut data have a strong crossover of chemicals that are used. As with the other data types, they all produce a similar result demonstrated by their respective MDS diagrams. C_6, hexane appeared to be present in males and females in similar amounts.

So, overall there are male / female differences within the samples from each different sanctuary group (Tamar and Bowes) and no male / female differences within the samples from the Chestnut sanctuary. These differences however were not observed when the Tamar and Bowes data was combined; instead there was a split in the data based on the different sanctuary groups. All differences (between sexes and sanctuaries) were demonstrated to be a possibly product of either digital or analogue coding (or a combination of the two).

Family membership coding in the North American beaver, *Castor canadensis* was shown to be a combination of both digital and analogue coding (Sun & Müller-Schwarze, 1998) and recognition of family members (using transferred family-specific scent) requires close and regular contact.

Similarly, the sub-caudal secretion of the European badger is used to scent mark the rump and flanks of conspecifics (Gorman et al, 1984; Hutchings & White, 2000; Buesching et al, 2002a), the ground around their sett and paths (Gorman et al, 1984; Buesching et al, 2002a), bedding material plus other objects and latrines (Buesching et al, 2002a). There have been many suggestions for the function of badger subcaudal secretion: territorial signalling (Kruuk, 1978a); group membership (Gorman et al, 1984); individual recognition (Kruuk et al, 1984) and fitness advertisement (Buesching & Macdonald, 2001).

European otters do not congregate in large family groups, living more solitary lives, so this type of allomarking is not carried out. In theory, family membership coding requires less information than individuality coding (Sun & Müller-Schwarze, 1998). The amount of variation in a signal must be equal or more equal to the amount of information the signal encodes ('the signal information match principle' – Sun 1996 in Sun & Müller-Schwarze, 1998). Therefore, the concept of digital and analogue coding may be extended to sexual and individual differences; however this may require a more complex mix of chemicals.

Part of the explanation for the differences observed between sanctuaries may be found in the European otter diet. Both sanctuaries (Tamar and Bowes) are run by the Otter Trust and there is a consistent dietary policy (see Materials and Methods, Table 1), however, the type of fish supplied to the otters is dependent upon availability. This could result in the differences seen in the spraint material (as this includes faecal as well as secretory elements). However, this result remains an odd one as it might be expected that any male / female message might be consistent regardless of the type of food an otter consumed.

Seasonal and locational changes in wild European otter diet may also, therefore, exert a significant influence over the olfactory make-up of otter spraints. Odour from dietary sources (due to its ever changing nature) would seem to be unlikely to contribute to individual identity; however, it may serve to identify animals that forage in a certain area or to distinguish between males and females (if there was a difference in feeding behaviour).

Fishstocks differ significantly, both within and between (UK) river catchments. Within most rivers there is a general trend of salmonids - rheophilic cyprinids -

eurytopic cyprinids from upstream to downstream, while the floodplain may support eurytopic and limnophilic species (Nunn, 2007).

Faecal odour in a spraint might therefore provide an indication to the receiver of the status of its depositor. For example, territory holders with exclusive access to superior feeding grounds may be partially identified by intruders via the intensity of the food odour in their scent marks. It may be that European otter status is a more important message than otter sex for an intruder and this could explain the sanctuary data split overriding any male-female split.

In mammals, learning, specialisation, social affiliation and status (changes with age and may differ between the sexes) all have an effect on diet at the individual level (Heggberget & Moseid, 1994).

European otter diet shows considerable variation in the composition of prey species (Chanin, 1985). Although European otters are largely piscivorous (Mason and Macdonald, 1986) they also demonstrate the ability to vary their diet by taking terrestrial prey (Weber, 1990).

Studies on captive European otters have shown indications of freshwater prey selection (mobile prey was preferred to more immobile prey and larger prey preferred to smaller prey) (Erlinge, 1968). Very few studies have investigated the influence of sex, age or individual preferences on the European otter diet. No significant differences between the stomach contents of male and female inland European otters were found (Erlinge & Jensen, 1981). However, sex and age was shown to affect the size and species composition of prey taken by marine-feeding otters on Shetland (Kruuk & Moorhouse, 1990). Male European otters are an average of 45% heavier than females (Chanin, 1985) and so will have a bigger energy requirement meaning that they take either more prey or larger prey. Female otters have been observed to catch large prey for their cubs and then consume small prey themselves whilst in the water (Kruuk et al, 1987), also indicated by the results of a study by Heggberget & Moseid (1994).

In addition, less experienced/skilled individuals may feed on prey that is easier to catch, but are less rewarding. For example, on Shetland, young European otters ate crabs more frequently than their adult counterparts (crabs are relatively east to catch although they a lower profit in terms of energy, due to their hard shell, than other

prey) (Watson, 1978). This was not supported by the results of the Heggberget & Moseid (1994) study on Norwegian otters. Sub-adult otter diet was found to be similar to that of adult European otters. This implies that these Norwegian European otters are not limited by food resources (Heggberget & Moseid, 1994)

If food resources are distributed in patches, social rank may govern the type and quality of prey an individual is able to obtain (Heggberget & Moseid, 1994).

Very few studies have investigated the presence of male / female differences in mustelid anal sac secretion.

The chemical profiles of the anal sac secretion of the stoat have been shown to include compounds common to both sexes plus one male specific compound (unknown non-suphur-containing compound) and two female specific compounds (2-ethylthietane and 3-ethyl-1,2-dithiolane) [Crump, 1980b].

Recent studies have confirmed that ferrets have the ability to distinguish between the odour of anal gland secretion in male and female conspecifics, however they are unable to do this at an individual level (Woodley & Baum, 2004).

Davies et al (1988) found no consistent sexual or individual differences in the anal sac secretion scent profiles of the European badger.

As well as intra-specific differences in the chemicals present in otter scent marks, there may also be inter-specific differences. This possibility was investigated in Chapter 5.

CONCLUSIONS

- Types of compound found in European otter spraints are typical of those found in the scent marks of other mustelid species.
- Spraint odour of European otters from different sanctuaries showed significant differences and this may be diet related.
- No overall male - female differences were found within the spraint odour, although within each sanctuary a possible difference between males and females was seen.

FUTURE WORK

- More of the compounds could and should be confirmed using comparison with synthetic material.
- An increase in the number of samples would help to further clarify any patterns in the data
- Perhaps a closer look needs to be taken at urine as a source of male / female differences.

CHAPTER 5

INTER-SPECIFIC DIFFERENCES IN SEMIOCHEMICAL ODOUR PROFILE OF FOUR MUSTELID SPECIES: THE EUROPEAN OTTER, *Lutra lutra*; THE CANADIAN RIVER OTTER, *Lontra canadensis*, THE ASIAN SHORT-CLAWED OTTER, *Aonyx cinerea* AND THE EUROPEAN BADGER, *Meles meles*

INTRODUCTION

Otters are members of the Mustelidae or weasel family (McDonald & King, 2000) and are typically solitary and territorial (Powell, 1979). Mustelids have diversified into highly specialized species with the ability to exploit a variety of contrasting environments (Neal & Cheeseman, 1996). Otters are no exception to this, but occasionally more than one species will occur in close proximity, and there is evidence for interaction leading to exclusion (Kruuk et al, 1994). Otters are found in all continents (except Australia and Antarctica) and any overlap in the range of different species is normally only observed along with major differences in diet or habitat (Kruuk et al, 1994). In South-East Asia three otter species occur sympatrically, the Asian short-clawed otter (*Aonyx. cinerea*), the European otter (*Lutra lutra*), and the smooth otter (*Lutra perspicillata*). Small differences in their use of riparian habitat and in diet have been observed, however there is also a degree of overlap (Kruuk et al, 1994). Sprainting behaviour in the European otter has been linked with home range advertisement and territorial marking (Erlinge, 1967b, 1968; Kruuk & Hewson, 1978; Macdonald 1980) as well as resource marking (Kruuk, 1995). Effective intra-specific communication through scent marking means an animal may be able to at least distinguish its own odour from that of another individual. This basic premise must also hold true for effective inter-specific communication (Brinck et al, 1983).

In this study we compare chemical composition in the headspace of freshly deposited scent marks of four mustelid species: the European otter the Canadian river otter (*Lontra canadensis*); the Asian short-claw otter and the European badger (*Meles meles*) in order to investigate similarities and / or differences in scent odour between species that could facilitate such a communication mechanism.

The European otter is one of the smaller otter species, but the largest of the 3 species examined in this study (see Table 1). It has a huge geographical distribution (larger than that of any other species). Its range stretches from the United Kingdom and Europe in the west, to the rainforests of Asia in the east and from Northern Africa, India and Indonesia up into northern Lapland and Siberia (Woodroffe, 1994). The European otter feeds primarily on fish, which makes up 70-95% of their diet (Woodroffe, 1994), but they also known to prey upon invertebrates, amphibians, reptiles and small mammals (Chanin, 1993), rabbits, water birds or even ducks

(Kruuk, 2006). They live and forage in both fresh and saltwater and their feeding behaviour is dependent upon local prey availability and catchability (Mason & Macdonald, 1980; Erlinge, 1967a). In freshwater habitats, the European otter is normally nocturnal, in contrast with coastal populations where activity is diurnal. This difference in behaviour can be explained by the variation in activity pattern of the main prey species (see Chapter 1, for further detail). The European otter is a solitary animal and largely nocturnal; its vocal and visual signs are poorly developed compared with more sociable otter species, e.g. the Asian short-claw otter and the giant otter, *Pteronura brasiliensis* (Trowbridge, 1983). Spraints are deposited on small rocks, logs or patches of grass, which may be permanent sprainting stations (Kruuk, 2006). The spraints are usually small for the size of the animal, with the larger males depositing dozens of tiny ones on a daily basis (Kruuk, 2006).

The Asian short-claw otter is the smallest otter in the world (Stone, 1992) - see Table 1 for comparison to the other species examined in this study. It has a distribution stretching from the Philippines through Indonesia, south-east Asia, southern China, and west through the Himalayan foothills of Bangladesh, Bhutan and Nepal plus in an isolated population in southern India (Stone, 1992). The Asian short-claw otter lives and forages in mangrove swamps and freshwater wetlands, feeding primarily on molluscs, crabs and other small aquatic animals (Stone, 1992). Occasionally, this species will also take frogs or fish; however they seem to be the least piscivorous of all the otter species (Kruuk, 2006). Its forepaws are only partially webbed and have claws that do not extend above the end pads. This gives them the dexterity required to forage in mud and under stones for their prey. Asian short-claw otter are highly social animals and live in groups of up to 15 individuals (Stone, 1992), with an average group comprising 5 individuals (Kruuk, 2006). They have a large repertoire of calls compared to less sociable species such as the European otter (Chanin, 1993). The short-clawed otter, despite its gregarious nature, is not a cooperative hunter and each individual will forage independently. It deposit spraints, which have conspicuous white pieces of crab carapax. In addition, they are larger (8 cm long and 3 cm in diameter) than those from other otter species sharing the same environment (Kruuk, 2006). Often, they are deposited away from the water and in the hollows between boulders rather than at a high point.

The Canadian river otter is a similar length to the European otter, but is more slender (see Table 1). It was once found throughout North America, from Arctic Alaska to the soutern states of Florida and Texas, however, over the last century its range has significantly reduced. Water pollution, habitat destruction and hunting have all contributed to its absence from much of the United States interior and the southern Canadian prairies (Stone, 1992). Like the European otter, it is a solitary animal and lives and forages in both fresh and saltwater, feeding mainly on fish, supplemented with invertebrates, amphibians, reptiles and small mammals (Stone, 1992) as well as birds (Kruuk, 2006). Despite often being alone, the Canadian river otter has been observed in groups made up of up to eighteen individuals, although three or four individuals is more normal (Kruuk, 2006). Communication through scent marking is utilised. Heaps of moss and soil are scraped together providing a visual sign, but probably also convey an olfactory message due to scent glands on the underside of the otter's feet (Kruuk, 2006). More in evidence are collections of strong smelling spraints (these smell very different to those of *L. lutra*) and are found closely associated with large dens that include freshwater for drinking and washing (Kruuk, 2006).

All three otter species belong to different genera (*Lutra*; *Lontra*; and *Aonyx*) and all are different sizes and weights (see Table 1).

Table 1. Comparison of 3 otter species

	Range	Main prey	Approx weight (kg)	Approx. length (cm)
European otter	Eur/Asia/Afr	Fish	8-10	110-120
Canadian river otter	N & S America	Fish	7-9	110-120
Asian short-clawed otter	Asia	Inverts.	4-5	100-115

Lengths and weights given for males; females are approximately 10% shorter and 25% lighter. Information taken from Chanin (1993).

NB only 2 of the otters in this study (the European otter and the Asian short-claw otter) exhibit any range overlap, e.g., these species occur sympatrically in Thailand (Kruuk et al, 1994).

The European badger *Meles meles* is a highly adaptable, medium-sized carnivore, distributed throughout temperate Eurasia, which shows a wide diversity of social and spatial organization (Newton-Cross et al, 2007). The badger is a true omnivore as it

consumes a wide range of animal and plant food. However, where readily available (e.g., in Britain, Ireland and much of Western Europe), the badger will primarily forage for earthworms (Neal & Cheeseman, 1996). In the UK they are also a predator of the western European hedgehog, *Erinaceus europaeus* (Doncaster, 1992). Badgers are mostly crepuscular and nocturnal; they lead a subterranean existence and can dig out extensive burrow systems (setts). These provide shelter during the day and are also used for breeding (Neal & Cheeseman, 1996). In Britain, badgers commonly live in large groups (mean group size – 5-6 individuals, Roper et al, 1993) comprising both males and females (Woodroffe & Macdonald, 1993; Neal & Cheeseman, 1996). In other areas of their range, studies have demonstrated badgers living alone or in pairs with intra- *or* inter-sexual territoriality (Woodroffe & Macdonald, 1993). Badgers defend territories usually not more than $1km^2$ (Roper et al, 1993). They defecate and urinate at latrines, which occur at territory boundaries and in the hinterland. Also, some studies have shown that where population density is low a substantial percentage of faecal depositions are left singly (Hutchings, 1996). Badger's anal glands produce an orange strong-smelling secretion and this is often deposited along with faeces at latrines (Neal, 1977; Roper et al, 1986). It has been suggested that this secretion also plays a role in territorial demarcation (Neal, 1977; Kruuk, 1978a; Roper et al, 1986).

As in Chapter 4, this study used freshly deposited spraints and scats, which included faecal material in addition to anal sac secretion alone. The study used solid phase micro extraction (SPME) and gas chromatography – mass spectrometry (GCMS) to investigate the chemicals present in the scent marks and whether any species specific patterns exist, either through unique sex specific / individual scent compounds or differing relative proportions of the same scent components.

MATERIALS AND METHODS

Study animals and sample collection

Spraint and scat samples were collected from 13 captive mustelids (4 different species).

Spraint and scat samples were collected at:

- ***The Chestnut Centre***, Derbyshire, UK (privately owned otter and owl sanctuary):
    - 4 captive-bred European otters (2♂/2♀). Three spraint samples from consecutive days were collected from each animal.
    - 4 captive-bred Asian short-claw otters (2♂/2♀). Single spraint samples were taken from each animal.
    - 3 captive bred Canadian river otters (1♂/2♀). Again, single spraint samples were taken from each animal.

- ***Mablethorpe Animal Gardens***, Lincolnshire, UK:
    - 2 European badgers at Mablethorpe Animal Gardens in Lincolnshire, UK. Single scat samples were taken from each animal.

Spraint and scat samples were collected 'immediately' following deposition – this was achieved by careful and patient observation of the animals (see Chapter 8). Each sample was placed individually into labelled airtight glass containers. The samples were stored over dry ice and immediately transferred to -70°C upon arrival at the laboratory to avoid deterioration prior to analysis.

NB Spraints were generally deposited near the corners and at the pen boundaries, although they were also found near the freshwater pools and lying-up shelters.

Three spraint samples from consecutive days were collected from each captive European otter Single spraint samples were collected from each member of the other species. These samples were supplemented with 3 spraints from unknown wild individuals from an established scent-marking site on the river Hull at Wansford, East Yorkshire, UK [grid reference: (TA)064560]. All sample collection was carried out in

August, 2002. The captive spraint samples were collected immediately following deposition and placed individually into labelled airtight glass containers. The site for the wild European otter samples was cleared of all spraints at dusk and freshly deposited spraints were collected the following morning. All samples were stored over dry ice. The samples were immediately transferred to -70°C upon arrival at the laboratory to avoid deterioration.

Sample preparation and headspace analysis

Sub-samples were placed into 4ml amber glass vials (providing a consistent ratio of headspace: sample of 1:1). The headspace of each was extracted with solid phase micro-extraction (SPME) using a 100µm polydimethylsiloxane (PDMS) fibre (Supelco, Bellefonte, PA) - used for low molecular weight / volatile compounds. Exposure duration was 20 minutes allowing equilibration and providing maximum adsorption of odour chemicals. The fibre was then introduced into the GC injection port for desorption (5 minutes).

GC analysis

Fibre contents were analysed by capillary GC-MS, with a CE Instruments HRGC 8000 Gas Chromatograph coupled with a Finnigan MD800 Mass Spectrometer, controlled by a Finnigan MD Family GC-MS Data System. The column was a DB-5MS column (J & W 30m, 0.316mm I.D., 0.25µm coating) with helium (1ml/min) as carrier gas.

Temperature program: 40°C held for 5 min, followed by a 5°C/min ramp up to 150°C held for 2 min, followed by a further 15°C/min ramp up to 270°C. A splitless injection was used with an injection temperature of 260°C. Fibre desorption time was 5 min.

Relative retention times and peak fragmentation spectra obtained by GC-MS were analysed using Masslab (Finnigan, San José, CA, USA). Any peaks from the column/fibre (mainly siloxanes) and those from the empty vial control were discounted from the analysis. All substances were given tentative identification using the Finnigan MD Family GC-MS Data System NIST library (the best match was chosen in each case). NB: occasionally chemicals were listed as a type of chemical

(e.g., alkane / aldehyde). Where no close match could be found, the chemicals in question were labelled as '*??*'.

Total ion count (TIC) was used for each of the peaks on the resultant chromatograms, and these values were normalised to calculate the relative content of each compound in a sample. All peaks were used in the analyses.

Statistical data analysis

As in Chapter 4, Multi- Dimensional Scaling (MDS) [Primer 5, version 5.2.0] was employed to clarify any underlying trends in the relatedness of compound composition. This enabled comparison of scent marks from the 4 captive mustelid species involved (plus samples from wild European otters).

The MDS analysis enabled comparison of

- $\log_{10}$ transformation of 'absolute' or 'raw' data;
- 'presence or absence' data

These analyses were performed on:

- The spraint odour profiles of 4 captive mustelid species (European otter, Asian short-claw otter, Canadian river otter, European badger) and wild European otter.

SIMPER analysis was again applied to the similarity matrices generated for each scent mark group. This examined the contribution of each chemical to the average Bray-Curtis dissimilarity between the sample groups.

Analysis of similarities (ANOSIM) was also carried out using the PRIMER software. As described in chapter 4 this procedure provided a way to test statistically whether there was a significant difference between two or more groups of sampling units (in this case, different species of mustelid).

Once again, as in Chapter 4, in comparing the spraint profiles, the concept of `overlap' between samples was used.

The differences found between samples were tested for significance using independent *t*-tests. Levene's test for equality of variances was initially employed to identify the most appropriate t-test (equal variances assumed/equal variances not assumed).

RESULTS

In total 72 peaks were found, of which 10 were found in all samples and 54 were specific to a particular species (1 to captive European otter, 2 to wild European otter, 2 to Asian short-claw otter, and 49 to European badger). Canadian river otter had no unique peaks.

NB The initial part of the European otter odour profile was identical in both captive and wild samples implying that the differing spraint collection methods did not affect loss of high volatility molecules. The wild European otter odour profile had 6 more components than the captive European otter.

Volatiles and semi volatiles obtained directly (using SPME - 100µm polydimethyl-siloxane (PDMS) fibre - for volatiles, amines and nitro-aromatic compounds) from the spraint and scat material of 4 different mustelid species were analysed.

19 compounds were found in captive European otter spraint material, whilst the spraints of their wild counterparts contained a total of 25. Canadian river otter and Asian short-claw otter spraints contained 17 and 19 compounds respectively. In contrast, badger scat material contained a much larger number of compounds at 61. Further details regarding the nature of these chemicals can be found in Tables 2 - 6.

Table 2. 19 chemical compounds in captive European otter spraints

1cyclobutanol	**9** C6	**22** dihydro-3,5-dimethyl-2(3H)-furanone
3 isopropyl alcohol	**11** chloroform	**33** benzene-like compound
4 tetramethyloxirane	**12 trans-7-methyl-3-octene**	**37** benzene-like compound
5 C4	**13** 3-methyl-butanal	**41** benzaldehyde
6 methylene chloride	**14** 2-methyl-1-nitro propane	**56** (E)-2-heptenal
7 o-(3-methylbutyl)-hydroxylamine	**15** 2,2-dimethyl hexane	
8 2,2,4,4-tetramethyl pentane	**16** 2-chloro-2-nitro-propane	

Table 3. 25 chemical compounds in wild European otter spraints

1cyclobutanol	**13** 3-methyl-butanal	**36** tetrahydro-3,6-dimethyl-2H-pyran-2-one
3 isopropyl alcohol	**14** 2-methyl-1-nitro propane	**37** benzene-like compound
4 tetramethyloxirane	**15** 2,2-dimethyl hexane	**39** heptanal
5 C4	**17 1-tert-butoxy-2-methoxyethane**	**41** benzaldehyde
6 methylene chloride	**22** dihydro-3,5-dimethyl-2(3H)-furanone	**46** 2,6,8-trimethyl-decane
7 o-(3-methylbutyl)-hydroxylamine	**28** o-pentyl-hydroxylamine	**50 octanal**
8 2,2,4,4-tetramethyl pentane	**30** hexanal	**65** C11
9 *C6*	**33** benzene-like compound	**71** C17
11 chloroform		

Table 4. 17 chemical compounds in captive Canadian river otter spraints

1cyclobutanol	**9** C6	**33** benzene-like compound
3 isopropyl alcohol	**11** chloroform	**37** benzene-like compound
6 methylene chloride	**13** 3-methyl-butanal	**41** benzaldehyde
7 o-(3-methylbutyl)-hydroxylamine	**15** 2,2-dimethyl hexane	**56** (E)-2-heptenal
8 2,2,4,4-tetramethyl pentane	**16** 2-chloro-2-nitro-propane	**65** C11
	22 dihydro-3,5-dimethyl-2(3H)-furanone	**70** indole

Table 5. 19 chemical compounds in captive Asian short-claw spraints

1cyclobutanol	**9** C6	**33** benzene-like compound
2 trimethylamine	**11** chloroform	**41** benzaldehyde
5 C4	**13** 3-methyl-butanal	**46** 2,6,8-trimethyl-decane
6 methylene chloride	**14** 2-methyl-1-nitro propane	**70** indole
7 o-(3-methylbutyl)-hydroxylamine	**15** 2,2-dimethyl hexane	**71** C17
8 2,2,4,4-tetramethyl pentane	**16** 2-chloro-2-nitro-propane	**72 2-nonadecanone**
	22 dihydro-3,5-dimethyl-2(3H)-furanone	

Table 6. 61 chemical compounds in wild badger scats

1cyclobutanol	**28** o-pentyl-hydroxylamine	**49 3-methyl-tridecane**
4 tetramethyloxirane	**29 oxanamide**	**51 1-nitro-piperidine**
5 C4	**30** hexanal	**52 1,3-dichlorobenzene**
6 methylene chloride	**31 phospholane**	**53 acetic acid hexyl ester**
7 o-(2-methylpropyl)-hydroxylamine	**32 2-octenal**	**54 limolene**
8 2,2,4,4-tetramethyl pentane	**33** benzene-like compound	**55 eucalyptol**
9 *C6*	**34 fluoro-cyclohexane**	**57 3-octen-2-one**
10 isopropyl alcohol	**35 phospholane**	**58 ?**
11 chloroform	**36** tetrahydro-3,6-dimethyl-2H-pyran-2-one	**59 diethyl-benzene**
13 3-methyl-butanal	**37**benzene-like compound	**60 (E)-2-octenal**
14 2-methyl-1-nitro propane	**38 2-heptanone**	**61 1-(ethenyloxy)-3-methyl butane**
15 2,2-dimethyl hexane	**39** heptanal	**62 ?**
18 n-methyl-1-octadecanamine	**40 2-methyl-5-(1-methylethyl)-1,3-cyclohexadiene**	**63 1,3,3-trimethyl-bicyclo 2.2.1.heptan-2-one**
19 threonine	**41** benzaldehyde	**64 o-(3-methylbutyl)-hydroxylamine**
20 butanoic acid, methyl ester	**42 pinene-like compound**	**65** C11
21 butenedioic acid	**43 1,1-dichloro-2-hexyl-cyclopropane**	**66 n-nitro-n-octyl-1-octanamine**
23 o-pentyl-hydroxylamine	**44 3-carene**	**67 cis-1,3-cyclopentanediol**
24 dimethyl disulphide	**45 (Z)-2-octen-1-ol**	**68 2-methyl-propanoic acid, methyl ester**
25 o-(3-methylbutyl)-hydroxylamine	**47 6-methyl-5-heptene-2-one**	**69 quinoline**
26 ?	**48 6-bromo-2-hexanone**	**70** indole
27 ?		

NB **numbers** denote the peak numbers when the results from all chromatograms (for all 3 otter species plus the European badger) are considered together – see Table 7. Compounds in bold are species-specific.

Many of the chemicals found occur in more than one species, some occur in all species, some occur in 2 or more species, and a small number are, at least within the context of this study, 'species-specific' (See Figure 1 for examples). European otter spraints have 3 compounds that are species-specific; one appeared in the captive samples (**12 trans-7-methyl-3-octene)** and the others in the wild samples (**50 octanal** and **17 1-tert-butoxy-2-methoxyethane**). Captive Asian short-claw spraints also have 2 species-specific compounds (**2 trimethylamine** and **72 2-nonadecanone**). Captive Canadian river otter spraints have no species-specific compounds. In contrast to the otter spraints, the European badger scats contain a much larger number (43) of compounds that are exclusive (see Table 6). Table 7 highlights all similarities and differences between the 4 species.

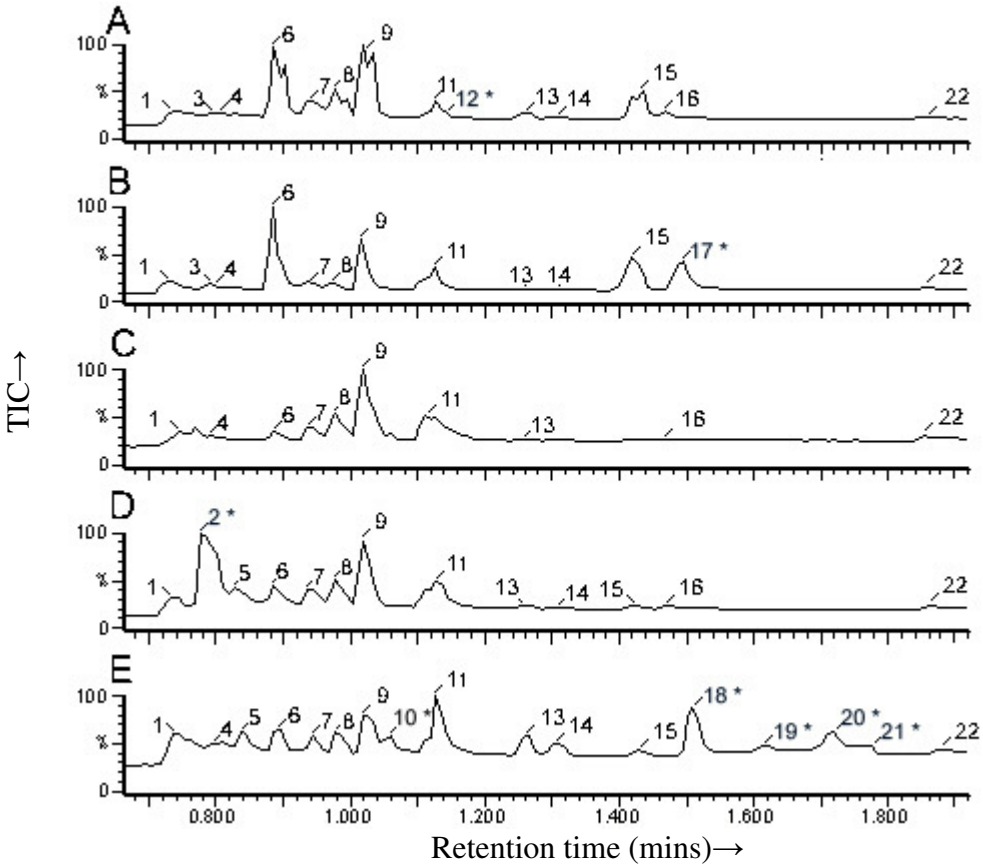


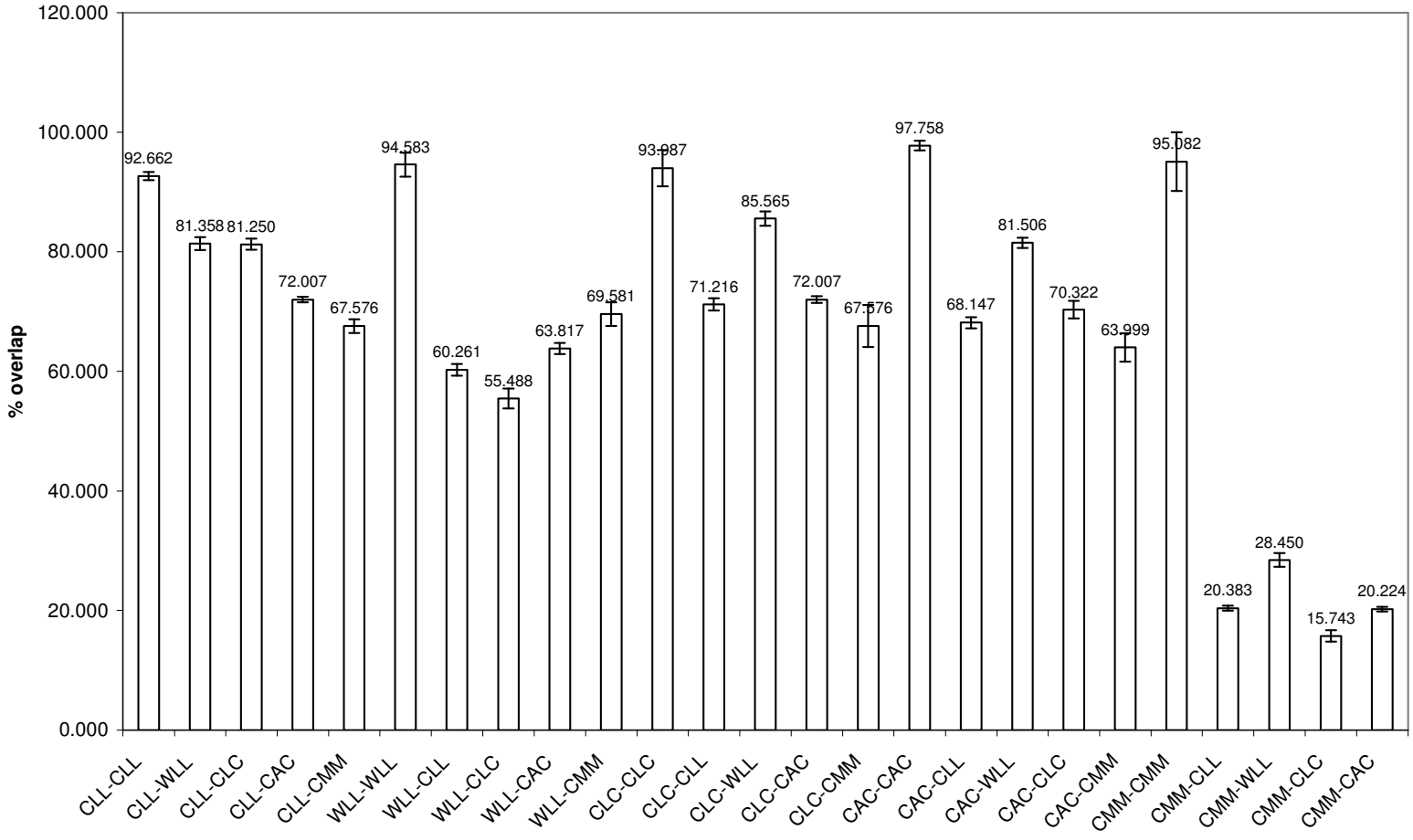
Figure 1. Chromatogram sections showing similarities/differences of compounds extracted by SPME from the spraints of 3 species of otter and the scats of the European badger. (A – captive European otter; B – wild European otter; C – captive Canadian river otter; D – Asian short-claw otter; E – European badger)

Most peaks in this section are found in every sample. 'Species-specific' compounds are denoted with an asterisk (*) – 2 *; 10 *; 12 *; 17*; 18 *; 19 *; 20 *; 21 *.

Table 7. Comparison of compounds found in spraint and scat material of 4 mustelid species

Peak number	Retention time (mins)	*L. lutra* (Captive)	*L. lutra* (Wild)	*L.canadensis* (Captive)	*A.cinerea* (Captive)	*M. meles* (Captive)
1	0.744	✓	✓	✓	✓	✓
2	0.777				✓	
3	0.785	✓	✓	✓		
4	0.811	✓	✓			✓
5	0.827	✓	✓		✓	✓
6	0.886	✓	✓	✓	✓	✓
7	0.944	✓	✓	✓	✓	✓
8	0.977	✓	✓	✓	✓	✓
9	1.019	✓	✓	✓	✓	✓
10	1.077					✓
11	1.127	✓	✓	✓	✓	✓
12	1.136	✓				
13	1.269	✓	✓	✓	✓	✓
14	1.311	✓	✓		✓	✓
15	1.419	✓	✓	✓	✓	✓
16	1.469	✓		✓	✓	
17	1.493		✓			
18	1.527					✓
19	1.636					✓
20	1.736					✓
21	1.786					✓
22	1.861	✓	✓	✓	✓	
23	1.894					✓
24	1.944					✓
25	2.036					✓
26	2.127					✓
27	2.194					✓
28	2.269		✓			✓
29	2.394					✓
30	2.803		✓			✓
31	4.12					✓
32	4.211					✓
33	4.345	✓	✓	✓	✓	✓
34	4.37					✓
35	4.595					✓
36	4.828		✓			✓
37	5.503	✓	✓	✓		✓
38	5.612					✓
39	6.087		✓			✓
40	7.112					✓
41	8.345	✓	✓	✓	✓	✓
42	8.837					✓
43	8.987					✓
44	9.079					✓
45	9.304					✓
46	9.371		✓		✓	
47	9.421					✓
48	9.512					✓
49	9.971					✓
50	10.104		✓			
51	10.104					✓
52	10.229					✓
53	10.529					✓
54	10.871					✓
55	10.971					✓
56	11.171	✓		✓		
57	11.379					✓
58	11.429					✓
59	11.821					✓
60	12.088					✓
61	12.171					✓
62	12.68					✓
63	12.98					✓
64	13.422					✓
65	13.755		✓			✓
66	13.738					✓
67	13.822					✓
68	16.639					✓
69	17.722					✓
70	19.523			✓	✓	✓
71	29.4		✓	✓	✓	
72	30.491				✓	

The result of the overlap analysis (discussed in the Materials and Methods section) can be seen in Figure 2.



[CLL – Captive *L. lutra*; WLL – Wild *L. lutra*; CLC - Captive *L. canadensis*; CAC – Captive A. cinerea; CMM – Captive *M. meles*]

Figure 2. Illustrates the difference in compound overlap between the 4 mustelid species. The mean overlap *within* each species was higher in every case than the *between* the species. The biggest contrast was seen in the mean overlap within the European badger compared with the mean overlap between the badger and the otter species.

Table 8. wild European otter and captive European otter, Canadian river otter, Asian short-claw otter, and badger scent mark chemical overlap - Independent Samples Test

	t-test for Equality of Means		
	t	df	Sig. (2-tailed)
captive European vs wild European	8.938	66.732	.000
captive European vs captive Canadian river	18.563	76.796	.000
captive European vs captive Asian short-claw	25.016	175.587	.000
captive European vs captive badger	18.859	41.485	.000
wild European vs captive European	13.705	40	.000
wild European vs captive Canadian river	15.063	13	.000
wild European vs captive Asian short-claw	16.084	16	.000
wild European vs captive badger	8.884	10	.000
captive Canadian river vs captive European	4.607	40	.000
captive Canadian river vs wild European	2.582	6.534	.039
captive Canadian river vs captive Asian short-claw	2.936	5.340	.030
captive Canadian river vs captive badger	7.404	10	.000
captive Asian short-claw vs captive European	15.360	58	.000
captive Asian short-claw vs wild European	13.972	22	.000
captive Asian short-claw vs captive Canadian river	16.565	17.130	.000
captive Asian short-claw vs captive badger	13.467	8.608	.000
captive badger vs captive European	15.133	1.015	.040
captive badger vs wild European	13.201	1.109	.037
captive badger vs captive Canadian river	15.832	1.078	.033
captive badger vs captive Asian short-claw	15.175	1.012	.041

As can be seen in Table 8, there is a significant difference between the mean overlap of the groups in each of the species comparisons (this is denoted by the p values – all are <0.05).

Comparison of spraints from different mustelid species using Multi-Dimensional Scaling (MDS)

Absolute content data, log_{10} transformed 1 way Global ANOSIM [R=0.932, p=0.001]

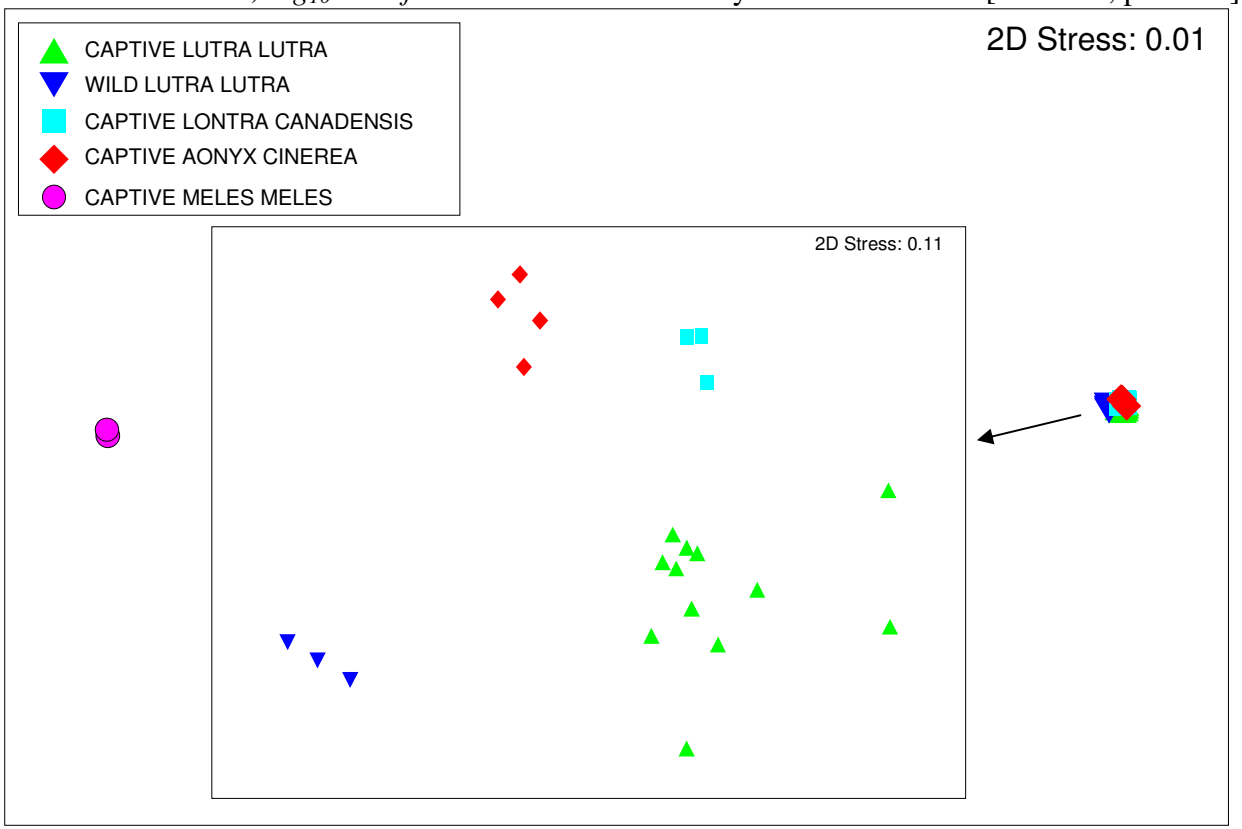


Figure 3. Multi Dimensional Scaling diagram showing relatedness between mustelid species, based on log_{10} transformed absolute scent mark odour chemical data. Global ANOSIM R value indicates an overall significant difference between the mustelid species.

Presence or absence data 1 way Global ANOSIM [R=0.987, p=0.001]

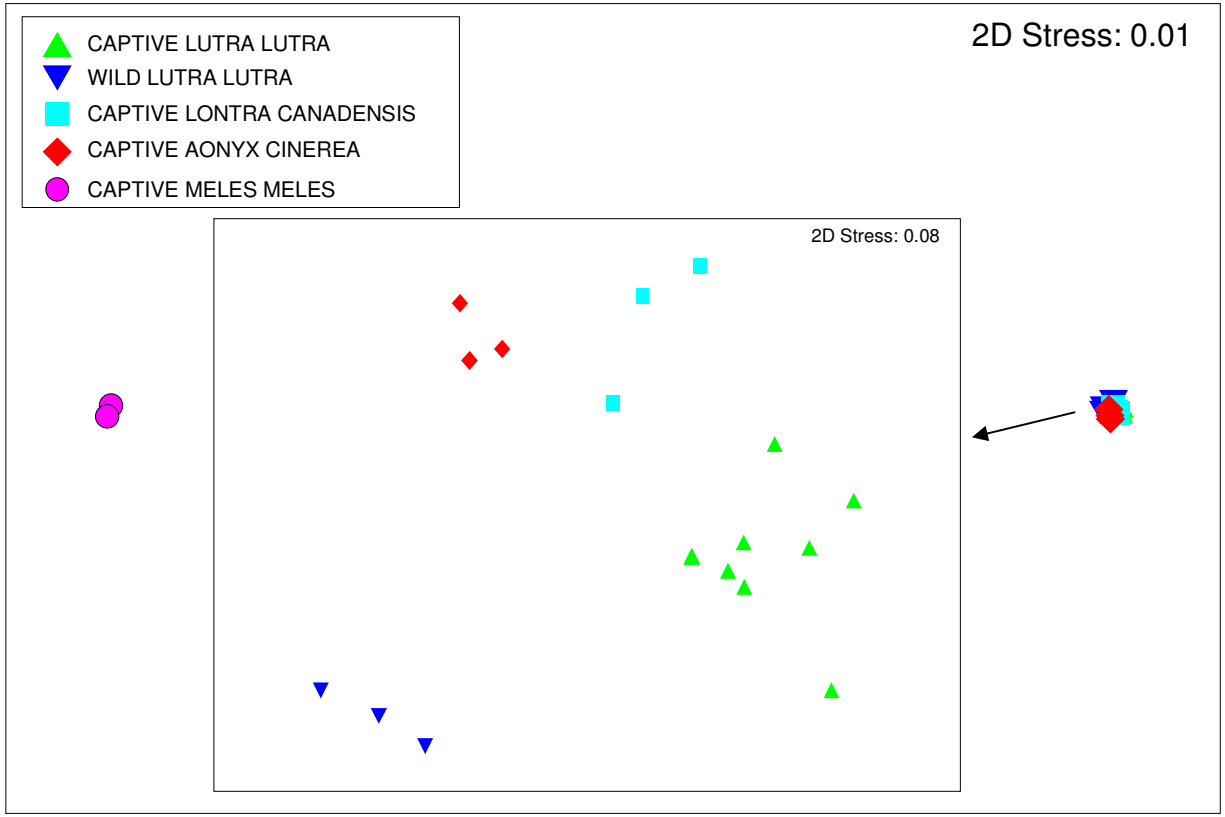


Figure 4. Multi Dimensional Scaling diagram showing relatedness between mustelid species, based on presence or absence scent mark odour chemical data. Global ANOSIM R value indicates an overall significant difference between the mustelid species.

Due to large differences between badger samples and the otter samples, the different otter species were tightly grouped in the in original full MDS plot. A subset MDS was derived using the cluster of samples for the otter species alone and overlaid on the original MDS to clarify similarities between individual otter species. Both MDS analyses using scent mark odour chemical data (absolute and presence or absence) showed a similar result; a definite separation between the 5 mustelid scent mark groups (captive European otter; wild European otter; captive Canadian river otter; captive Asian short-claw otter; captive badger). This was confirmed by the Global ANOSIM results for both data types (absolute and presence or absence). A significant overall difference between mustelid species was seen. Pairwise comparisons between separate pairs of mustelid species were also made. Table 9 shows the results of these comparisons for both data types.

Table 9. Pairwise Anosim comparison of mustelid species (*Abs* – Absolute data; *P/A* – Presence or Absence data)

Groups		R statistic		p value		Possible Permutations	Actual Permutations
		Abs	*P/A*	*Abs*	*P/A*	(for both data types)	(for both data types)
1	Captive European, Wild European	0.99	1	0.002	0.002	455	455
2	Captive European, Captive Canadian river	0.77	0.95	0.002	0.002	455	455
3	Captive European, Captive Asian short-claw	0.92	0.99	0.002	0.001	1820	999
4	Captive European, Captive badger	1	1	0.011	0.011	91	91
5	Wild European, Captive Canadian river	1	1	0.1	0.1	10	10
6	Wild European, Captive badger	1	1	0.1	0.1	10	10
7	Wild European, Captive Asian short-claw	1	1	0.029	0.029	35	35
8	Captive Canadian river, Captive Asian short-claw	1	1	0.029	0.029	35	35
9	Captive Canadian river, Captive badger	1	1	0.067	0.067	15	15

All results show a significant difference between each pair of mustelid species. NB Significance is influenced by the number of permutations and this is particularly relevant if the number of possible permutations is low. Some of the comparisons above had particularly low numbers of permutations, for example - comparisons 5 and 6. These comparisons had an R value of 1 (indicating a large difference between species), but a p value of 0.1. Here, the significance level may be misleading as there are only 10 permutations possible so significance can never get above p=0.1 regardless how strong the difference. Conversely, if a high number of permutations is seen then you may get a significant result in terms of the p value, but if R is small then this indicates a relatively minor or trivial (albeit significant) difference.

Further analysis was now carried out in order to ascertain which chemicals might be most responsible for the separation seen. Tables 9 and 10 summarise the % similarity within and the dissimilarity between the sex groups for each data type. Tables 11 and 12 show the chemicals most responsible for these similarities and dissimilarities.

SIMPER analysis

Similarity / dissimilarity summary tables

Table 10 summarises the the average percentage similarities within the mustelid species scent mark groups

Table 10. Summary of average percentage similarity within the mustelid species groups in terms of spraint/scat odour profile.

Data type	Average % Similarity				
	captive European	wild European	captive Canadian river	captive Asian short-claw	captive badger
absolute content data, log_{10} transformed	82.97	93.18	92.10	91.46	92.40
presence or absence data	92.00	95.74	93.54	97.27	94.83

Each scent mark odour chemical profile within each species group has a high average percentage similarity.

Table 11. Summary of average (%) dissimilarity between mustelid species groups in terms of scent mark odour, based on log_{10} transformed absolute and presence or absence spraint/scat chemical data.

	captive European otter	wild European otter	captive Canadian river otter	captive Asian short-claw otter
wild European otter	40.57 35.90	-	-	-
captive Canadian river otter	27.78 24.38	42.5 37.96	-	-
captive Asian short-claw otter	33.94 30.45	39.07 32.83	27.14 23.21	-
captive badger	71.97 68.81	61.25 58.82	76.43 75.13	72.68 69.34

Table 11 shows that for both the data types the captive badger scent marks are relatively dissimilar to all of the otter species scent marks. Also, all captive species scent marks are less dissimilar to each other than they are to the wild scent marks.

Table 12 summarises the dominant chemicals responsible for similarities within species scent mark groups.

Table 12. summary of odour chemicals with the largest contribution (%) to similarity within the species groups

Data type	Average % Similarity				
	captive European	wild European	captive Canadian river	captive Asian short-claw	captive badger
absolute content data, log_{10} transformed	C_6, hexane (8.53)	chloroform (5.13)	cyclobutanol (8.52)	trimethylamine (7.19)	tetrahydro-3,6-dimethyl-2H-pyran-2-one (2.38)
presence or absence data	(E)-2-heptenal (6.26)	C11 (4.42)	(E)-2-heptenal (6.98)	indole (5.56)	eucalyptol (1.82)

Interestingly, inter-specifically (rows), each species has a different dominant chemical (in terms of average similarity) in each of the data categories. Intra-specifically (columns), each species also has a different dominant chemical in each data category.

Table 13 summarises the dominant chemicals responsible for dissimilarities within species scent mark groups.

Table 13. Summary of average dissimilarity between mustelid species groups in terms of scent mark odour, based on log_{10} transformed absolute and presence or absence spraint/scat chemical data.

	captive European	wild European	captive Canadian river	captive Asian short-claw
wild European	C_{11} (7.14) (E)-2-heptenal (6.8)	-	-	-
captive Canadian river	indole (11.28) indole (12.57)	C_{11} (7.14) (E)-2-heptenal (6.77)	-	-
captive Asian short-claw	trimethylamine (10.36) (E)-2-heptenal (9.16)	trimethylamine (7.49) C_{11} (7.23)	trimethylamine (10.36) (E)-2-heptenal (12.76)	-
captive badger	tetrahydro-3,6-dimethyl-2H-pyran-2-one (2.41) eucalyptol (1.93)	type of pinene (2.54) eucalyptol (2.08)	tetrahydro-3,6-dimethyl-2H-pyran-2-one (2.33) eucalyptol (1.82)	tetrahydro-3,6-dimethyl-2H-pyran-2-one (2.32) eucalyptol (1.89)

Table 13 shows that the captive badger group is consistent in terms of the types of chemical most responsible for its dissimilarity with other species groups (log_{10} absolute: tetrahydro-3,6-dimethyl-2H-pyran-2-one (in all but the comparison to wild European otter); presence or absence: eucalyptol)

Captive Asian short-claw otter is also fairly consistent with trimethylamine dominating the log_{10} absolute data comparisons and (E)-2-heptenal dominating the presence or absence data comparisons with other otter species (apart from the comparison to wild European otter. - C_{11}).

A similar situation exists with comparisons involving the wild European otter samples. C_{11} dominating the log_{10} absolute data comparisons and (E)-2-heptenal dominating the presence or absence data comparisons with other otter species.

Comparisons involving both captive Canadian river otter and captive European otter had more variety in the dominant chemicals observed, however, (E)-2-heptenal dominates their presence or absence data comparisons with other otter species.

DISCUSSION

If inter-specific communication through scent marking is to work effectively then an animal must, at least, be able to recognize the odour of its own species. Certainly to the human nose, each of the species in this study could be distinguished from their scent marks, e.g., the distinctive odour of European otter spraints was in stark contrast to that of Asian short-claw otters, which smelt far more objectionable, whilst the odour of the Canadian river otter fell between the two, possessing elements of both. In a similar way, the strong musky odour of badger scat was instantly recognisable. All this suggests the possibility that one of the basic functions of spraint / scat odour may be to signal species identity. This could be achieved either through unique scent components (digital coding) or a 'blend' of common scent components (analogue coding) or even a combination of the two. This study showed that although there is a measure of overlap between the peaks in the odour profile of different species, most also possess unique peaks (see Figure 1). Of the 71 peaks found, 10 occurred in all species and 54 were unique (43 in badger; 3 in European otter, 2 wild, 1 captive; 2 in Asian short-claw otter and none in Canadian river otter). One explanation for the 10 common peaks could be that they represent an overall 'mustelid marker' and this might then be supplemented by different blends of chemicals together with unique chemicals making up a 'species marker' (see Table 13). It might be of course that unique chemicals may be enough to identify a species (digital coding), as in the case of European otter, Asian short-claw otter and badger, however, the Canadian river otter has no unique chemicals and so would have to rely solely on a unique *blend* of chemicals (analogue coding).

Table 14. Possible mustelid and species markers

Mustelid markers?	**Species markers?**					
	European	***Asian short-claw***	***Canadian river***	***Badger***		
Common peak numbers	Unique peak numbers					
1				10	43	64
		2		18	44	65
6				19	45	66
7				20	47	67
8				21	48	68
9				23	49	69
11				24	51	70
	12 (captive)			25	52	
13				26	53	
15				27	54	
				29	55	
	17 (wild)			31	57	
				32	58	
33				34	59	
41				35	60	
	50 (wild)			38	61	
				40	62	
		72		42	63	

The scats of the badger were shown to be more distinct from the otter species in terms of the chemicals they contained, due mainly to the significant number of 'species-specific' compounds present in comparison with the otters. As badgers (Melinae) and otters (Lutrinae) are members of different mustelid subfamilies (Brinck et al, 1983), this result is perhaps not unexpected.

Examining the otter species in isolation reveals a narrower range of 31 chemicals of which 13 occur in all three species. These 13 chemicals include the 10 found in all the samples (see Table 14), plus three more that only occur in the otter species (peak numbers 16, 22, and 71). In the same way that the 10 peaks might represent an overall 'mustelid marker', the 3 extra peaks may give another layer of odour representing an overall 'otter marker'.

Although the European otter has 3 unique chemicals in its spraints, one is found in the captive samples and the other two are found in the wild samples. As spraints contain faecal material as well as secretion from the anal glands, and as wild otters will consume a different diet to those that are captive, it is possible that the unique chemicals in the wild spraints are derived from the diet. These cheicals may be important, for example, in the identification of territory holders with exclusive access to superior feeding grounds. It is also possible that odour from faecal

material in scent marks may help to differentiate between species that have dietary separation.

Otters have been shown to be drawn to the scent marks of conspecifics in preference to those from other species. For example, in a study of North American river otters (Rostain et al, 2004) olfactory signals were suggested to communicate species and sexual identity. Coastal wild-caught adult male otters underwent a series of preference tests and were found to spend more time investigating spraints from conspecifics than those from sealions. Similarly, in marsh living river otter populations, otters visit conspecific scent in preference to that of mink, *Mustela vison* (Humphrey & Zinn, 1982).

Comparisons using Multi – Dimensional Scaling (MDS)

All MDS analyses for both data types indicate that the composition of the spraint chemical odour profile in the 4 captive species groups (plus 1 wild species European otter group) is different. Definite separation and close clustering of the 5 groups was seen with larger differences being observed between badger samples and the otter species samples. A significant overall difference was seen between all 5 sample groups, together with significant differences between each individual pair of mustelid samples (including the wild vs captive European otter sample groups).

Table 11 shows that each mustelid species had a different dominant chemical (in terms of average similarity) for each of the 5 mustelid categories. This is a further indication of the dissimilarity between the species in terms of their scent mark chemicals. Intra-specifically (columns), each species also had a different dominant chemical in each data category. Therefore, although different chemicals had varying levels of importance depending on the data type used, they all produced a similar result as demonstrated by their respective MDS diagrams.

Captive badger was shown to be relatively consistent in terms of the type of chemical most responsible for its dissimilarity to other species (absolute content: tetrahydro-3,6-dimethyl-2H-pyran-2-one (in all but the comparison to wild *L. lutra*); presence or absence: eucalyptol).

Each chemical for each data type was observed to be present in the captive badger samples, but not in any of the captive otter species (digital coding). NB tetrahydro-

3,6-dimethyl-2H-pyran-2-one was also found in the wild European otter samples (this is an example of analogue coding).

Trimethylamine dominated the absolute content data comparisons between captive Asian short-claw and the other otter species, whilst (E)-2-heptenal dominated the presence or absence data comparisons with other otter species.

Each chemical for each data type was observed to be present in the captive Asian short-claw samples, but not in any of the other otter species (digital coding). NB apart from (E)-heptanal, which was also found in the captive European otter samples (possible example of analogue coding, although in this case the levels did not appear to be that different in either species).

A similar situation existed with comparisons involving the wild European otter samples. C_{11} dominating the absolute content data comparisons and (E)-2-heptenal dominating the presence or absence data comparisons with other otter species.

C11 was present in the wild European otter samples, but not in any of the other mustelid sample apart from captive badger. Octanal was seen in the wild European otter samples, but not in any of the other mustelid sample. Conversely, (E)-2-heptenal was not present in the wild European otter samples, but was present in both the captive European otter samples and the captive Canadian river otter samples (all examples of digital coding).

All the chemicals examined so far have been present in some, but not all of the species. In order to illustrate the influence a chemical that occurs in every sample may have, benzaldehyde was chosen.

Levels of benzaldehyde for the absolute content data varied slightly between each species group (examples of analogue coding). NB obviously, the levels of benzaldehyde will all appear identical in every sample for the presence or absence data.

So it has been demonstrated that the mustelid species examined in this study have the potential to be discriminated between using their scent mark odour chemicals. This may be achieved via digital or analogue coding or even via a mechanism where both types are being employed.

As with the male / female differences seen in Chapter 4, otter dietary intake may partly explain differences in scent mark chemicals between different mustelid species. Here, most of the samples taken were from animals in a captive situation, the exception being those samples collected from wild European otter. In addition, all the captive otter species were from the same sanctuary (The Chestnut Centre, Chapel-en-le-Frith, Derbyshire). Table 15 summarises the diet of each mustelid species.

Table 15: comparison of mustelid species diet

Mustelid species	Diet
captive European otter captive Canadian river otter	Dead, day-old chicks; fish (variety of species; knackersmeat mixed with vegetables and a multivitamin (sometimes oats). Suet was given in winter. In addition these species were seen to also catch rats, mice and bullheads.
captive Asian short-claw otter	Dead, day-old chicks; trout and herring. They were also given crabs, grapes and peanuts in the summer. In addition, *A. cinerea* were known to catch invertebrates including earthworms.
captive badger	ZF6 (zoo food – food for wild animals, based on kitten food, with rabbit, chicken and herbs) supplemented with fruit, nuts and oats. They were also seen to catch earthworms (their primary food source in the wild), plus other invertebrates (caterpillars, moths and beetles) as well as the occasional mouse or rat.
wild European otter	Mainly fish - including brown trout, *Salmo trutta* L., juvenile salmon, *Salmo salar*, bullheads, *Cottus gobio*, minnows, *Phoxinus phoxinus* (Britton, et al, 2005), chub *Leuciscus cephalus* and perch *Perca fluviatilis* (Copp & Kováč, 2003) but can also include birds, insects, frogs, crustaceans and sometimes small mammal

All the species examined therefore (with the exception of captive European otter and captive Canadian river otter) had slightly different diets with that of the badger being the most different as it was the only one that included no fish. This may partly explain why the badger scent marks were so different to the other (otter) species in this study. Diet may also explain why captive European otter was least dissimilar to Canadian river otter (identical diet) followed by captive Asian short-claw otter (partially similar diet), then wild European otter (again a partially similar diet) and finally captive badger (a much different diet). However, as spraints were being used in this study it was impossible to separate the contribution from faecal material and

that from anal sac secretion. A further study using anal sac secretion alone for each species would be useful in terms of pinpointing those chemicals provided 'directly' from the diet.

Inter-specific communication will only be important for those animals that share habitats and perhaps only then if there is any overlap in the use of resources. One example involves a study of three otter species, the Asian short-claw otter, the Eurasian otter, and the smooth otter (*Lutrogale perspicillata*), occurring sympatrically in the Huai Kha Khaeng river system, Thailand (Kruuk et al, 1994). Differences between habitat and food preferences were demonstrated, but clear overlap was also seen. All three species were attracted to the sprainting sites of the others, and this suggests the possibility that the intra-specific function of spraints, e.g., resource marking in order to space out foraging individuals also works at an inter-specific level. This would be of benefit to all individuals involved regardless of species.

The potential 'mustelid marker' and 'otter marker' chemicals identified in this study may go some way to explaining the interest of certain otter species in the scent marks of other mustelids or otters (as in the example above). The differences in the diet of these sympatric species may also, in a small way, help to discriminate between different species.

In addition, spraint positioning, size and appearance will also help to discriminate between spraints from different species. For example, two of the otters in this study (the European otter and the Asian short clawed otter) exhibit range overlap, occurring sympatrically in Thailand (Kruuk et al, 1994). These species exhibit markedly different spraint positioning, size and appearance (see Table 16).

Table 16. Comparison of otter (European / Asian short-claw) spraints

Otter species	European otter	Asian short-claw otter
Appearance	principally made up of fish bones and scales, crustacea shell, fur or feathers	conspicuous white pieces of carapax, due to the abundance of crab in their diet †
Shape	small tar-like smear containing few or no bones, to cylindrical droppings *	cylindrical droppings
Size	1cm in diameter and up to 10 cm in length *	8 cm long and 3 cm in diameter†
Positioning	prominent features of the landscape, (on top of conspicuous rocks near the water, under bridges, at tributary junctions) or at places of interest (small freshwater pools, near to holts) **	away from the water and in the hollows between boulders rather than at a high point†

* Corbet and Harris, 1991 / ** Kruuk, 1995 / † Kruuk, 2006

Inter-specific communication through scent marking can also have disadvantages, e.g. it may alert potential predators to an animal's presence. Or it could have the potential to modify the behaviour of the animal's prey species, in response to predator scent resulting in avoidance. For example, foraging effort of free ranging hedgehogs has been shown to diminish in response to badger odour (Ward et al, 1997) and water voles have been shown to avoid the odour of both mink and rat (Barreto & Macdonald, 1999). Another example involves the coexistence of stoat (*Mustela erminea*) and weasel (*Mustela nivalis*). These two mustelid species are sympatric over most of their ranges (Erlinge & Sandell, 1988) and although the weasel is half the size of the stoat, both predominantly consume the same diet (small rodents, lagomorphs, and birds) [Erlinge, 1975; 1981]. Weasels have been shown to be able to distinguish between their own scent and the scent of the stoat. They avoided stoat odour in encounter tests, whereas stoats did not react to weasel odour (Erlinge & Sandell, 1988). Field data also indicated avoidance: high vole density habitats occupied by stoats were avoided by weasels, however they occasionally moved into such areas when abandoned by stoats. Brinck et al (1983) demonstrated a chemical basis for discrimination between the weasel and stoat anal sac secretion, with both qualitative and quantitative differences in evidence.

CONCLUSIONS

- A measure of overlap between the peaks in the odour profile of different species of mustelid was identified. Most also possess many unique, 'species-specific' peaks
- The presence of certain common chemicals may represent a 'mustelid' or 'otter' odour
- MDS analyses demonstrated a differentiation in scent mark odour profiles based on species. Results show coding for this differentiation to possibly be a combination of both analogue and digital elements. If otters were able to detect these differences then this could provide a mechanism intra-specific communication.
- This differentiation was in part, attributed to dietary influences. All the species examined (with the exception of captive European otter and captive Canadian river otter) had slightly different diets with that of badger being the most different.

FUTURE WORK

- A repeat of the study using increased numbers of samples, more species and perhaps using a range of different fibre types may reveal more information.
- Analysis of anal sac secretion alone would also be useful in terms of pinpointing contributions made 'directly' to the scent mark odour by diet.
- More of the compounds could and should be confirmed using comparison with synthetic material.
- SPME has been used to reveal inter-individual variation in the volatile compounds found in scent marks of spotted hyenas (Hofer et al, 2001). Further development in the method is necessary to investigate its sensitivity to individual differences between scent marks within otter and other mustelid species, with a view to its possible use as a monitoring tool in the field.
- Behavioural studies to examine otter's responses to spraint volatiles.

CHAPTER 6

INTRA-SPECIFIC DIFFERENCES IN THE FATTY ACID PROFILE OF EUROPEAN OTTER, *Lutra lutra* SCENT MARKS

INTRODUCTION

A variety of fatty acids have been shown to be present in the anal sac secretions of many mammals. For example, short chain fatty acids have been identified in the anal sac secretion of the red fox, *Vulpes vulpes* (Albone & Fox, 1971); the Indian mongoose, *Herpestes auropunctatus* (Gorman et al, 1974; Gorman, 1976) and the giant panda, *Ailuropoda melanoleuca* (Hagey & Macdonald, 2003).

The anal sacs provide a suitable environment to support a rich microflora. Sebaceous and apocrine secretions once inside the sac act as substrates for the activity of bacterial populations, which have a substantial effect on the eventual nature and odour of the deposited scent mark (Albone, 1984). Bacterial activity is thought to be responsible for volatile organic compounds in, amongst many others, the anal sacs of the red fox. No bacteria were, however, found in the anal gland of the European mole, *Talpa europaea* (Buglass et al, 1995).

Olfactory encoding for individual identity has been observed in a range of mammalian species. Chemical analysis of scent secretions from some mammals has identified quantitative differences in the relative concentrations of chemicals of odours from individual animals. For example, the anal sac secretion of the Indian mongoose, contains six volatile carboxylic acids (acetic, propionic, *n*-butyric, iso-butyric, *n*-valeric and iso-valeric), the relative concentrations of which are unique for each individual animal (Gorman, 1976). The biological activity of fatty acids in the mongoose anal sac secretion was confirmed by the ability of test animals to distinguish between synthetic mixtures containing different ratios of fatty acids (Gorman, 1976).

Long-chain fatty acids have been identified, for example, in the anal sac secretion of the Egyptian mongoose, *Herpestes ichneumon* (Hefetz et al, 1984) and the red panda, *Ailurus fulgens* (Wood et al, 2003).

Deposited scent marks frequently comprise sebaceous gland products, e.g., sebum (made up of glycerides, free fatty acids, wax esters, squalene, cholesterol esters, and cholesterol). These substances act as carriers for more volatile active odour components, which are released more slowly from substances of higher polarity. The carrier substance also buffers the active odour against any effects from the surface substrate, ensuring the signal survives over a longer period of time (Bradbury & Vehrencamp, 1998).

It has been suggested that less volatile long chain fatty acids would be more suited to a long term signalling system and may have a role in territorial or home range marking (Davies et al,

1988; Hefetz et al, 1984; Wood et al, 2003). The question remains open upon the function of such fatty acids. Do these less volatile compounds carry an olfactory message or are they part of a carrier system for other, more volatile active odour components? Or could they have a dual function?

Fatty acids are also present in the anal sac secretion of some mustelid species (mink, *M. vison* – Sokolov et al, 1980; European badger, *Meles meles* – Davies et al, 1988). However, although the anal sac secretion of the European otter has previously been investigated (Gorman et al, 1978; Jenkins et al, 1981; Trowbridge, 1983), these studies did not reveal detailed information regarding chemical composition and none specifically investigated fatty acid content (although, fatty acid presence is confirmed by Gorman et al, 1978). One more recent study employed purge and trap plus GCMS analysis of anal sac secretion (Bradshaw et al, 2001); no fatty acids were identified.

In this study, the fatty acid constituents of European otter scent mark material has been examined, initially by identifying those present. As in Chapters 4 and 5 freshly deposited spraints were used, which include faecal material in addition to anal sac secretion. This would provide as much information as possible about the fatty acid make-up of a deposited scent mark odour, as an animal would encounter it in the wild.

Spraint material from wild European otters was also examined by way of a comparison with their captive counterparts.

Additionally, in order to rule out or highlight any contribution directly made by diet the fatty acid content of European otter anal sac secretion (obtained from road-kill animals) was investigated.

MATERIALS AND METHODS

Study animals and sample collection.

Captive European otter spraint material: A total of 13 spraint samples were collected from 6 captive adult otters (3 male and 3 female – see Table 1) all housed at 'The Chestnut Centre', Chapel-en-le-Frith, Derbyshire, UK (a privately owned otter and owl sanctuary). Two spraint samples were collected from each individual immediately following deposition (NB one female provided 3 samples). All samples were placed in airtight, labelled glass containers which were stored over dry ice in an insulated container and transported to the lab the same day. Once at the lab the samples were stored at -70°C immediately in order to avoid deterioration prior to analysis. All spraint samples were defrosted in order to take a sub sample for freeze-drying. Approximately 1.5g of material was removed from each spraint sample. All sub samples were refrozen to -70°C.

Table1. The Chestnut Centre European otter information

Pens	A	B	C
Otter	Alpha & Izzy	Lancelot & Damsel	Morse & Sheil
Sex	male & female	male & female	male & female
DOB	1994 & 1995	both 1997	1996 & 1997
Diet			
Fedding Times	Food		
morning	dead, day old chicks		
afternoon	fish (variety of species) + knackersmeat mixed with veg and a multivitamin (and sometimes oats). Suet given in winter. Also seen to catch rats, mice, bullheads.		

- All pens contained a deep pool of freshwater for swimming.

Wild European otter spraint material: A total of 5 spraint samples were collected from unknown wild European otters from an established scent-marking site on the river Hull at Wansford, East Yorkshire, UK [grid reference: (TA)064560]. The samples were collected over a period of a week. The site was visited at dusk and cleared of all old spraints. Upon re-visiting the site the following morning any new, fresh spraints were placed in airtight, labelled glass containers, which were stored over dry ice in an insulated container and transported to the lab the same day. Once at the lab the samples were stored at -70°C immediately in order to avoid deterioration prior to analysis. All spraint samples were defrosted in order to take a sub sample

for freeze-drying. Up to 1.5g of material was removed from each spraint sample. All sub samples were refrozen to -70°C.

Wild European otter anal sac secretion: A total of 5 samples of anal sac secretion were harvested from the anal sacs of 5 wild road kill European otters. 4 male (2 adult, 2 sub-adult) and 1 adult female. These were found between March and December 2000. The corpses were originally sent to Dr Adeline Bradshaw at [Llysdinam Field Centre, Cardiff University, UK] for post mortem licensed through the Environment Agency. The dead animals were collected as soon as possible following discovery; however, there was an inevitable delay between discovery and collection. Each cadaver was dissected immediately to remove any material needed for analysis. The cadaver was then stored at –18°C. The anal glands were mailed (stored over dry ice) to Hull University where they were immediately stored at -70°C in order to avoid deterioration prior to analysis. The anal glands were removed from the -70°C freezer and allowed to defrost to an ambient temperature of 4°C in a domestic refrigerator set aside for the purpose. Following this process the contents of each anal gland were manually expressed into airtight labelled glass containers. All samples were refrozen to -70°C.

All samples (captive spraints / wild spraints / wild anal sac secretion) were then placed in labelled containers with a parafilm seal: the seal was punctured to permit free gas exchange during freeze-drying. The samples were freeze-dried overnight and transferred to conical flasks and weighed.

Fatty acid extraction procedure

(NB this procedure was developed by Professor Colin Ratledge's Microbial Biochemistry Research Group at the University of Hull, East Yorkshire)

i. 100ml chloroform:methanol (2:1) mixture was added to each sub sample.

ii. The flasks were capped tightly with foil and shaken for 4 hours.

iii. Each mixture was passed through filter paper (grade number 1) into separating funnels and 25ml of 0.9% saline was added to the filtrate.

iv. The separating funnels were mixed very gently and then left for 30 minutes in order for the mixture to partition into 2 layers (NB the chloroform:methanol layer retains virtually all of the lipids). The bottom chloroform:methanol layer was then separated off and retained while the top layer of saline was discarded.

v. 50mls of distilled water was then added to each flask.

vi. Step iv. was repeated.

vii. A further 50mls of distilled water was added to each flask.

viii. Step iv. was repeated (left overnight before separation).

Each sample was evaporated to dryness in a rotary evaporator with a final addition of absolute ethanol to remove any remaining water. Each extract was taken up in di-ethyl ether and transferred to weighed, labelled vials and the di-ethyl ether was allowed to evaporate leaving behind the fatty acid residue. All vials were re-weighed and the mass of the fatty acid residue plus the (dry weight) percentage fatty acid content of each sample could then be calculated. The fatty acid residues were taken back up in chloroform. Esterification of the fatty acid residues was achieved by the addition of 100mls of trimethylsulfonium hydroxide (TMSH), drop-by-drop, to each vial. The solution was required to become clear. If the solution became cloudy at this point, they were left to stand and then if necessary more chloroform or TMSH was added. 1µl of each spraint sample extraction was injected into the GC.

GC analysis

All spectra were obtained using a Thermo-Finnigan GCQ plus Ion Trap Mass Spectrometer 70ev E1MS using control acquisition software (Thermo-Finnigan version 2.31 (October 1998, pc based).

Temperature programme

The following conditions were used: source Temp: 220°C, Transfer Line: 280°C, Injector Temp 260°C. Separation was performed on DB5-MS column (J & W 30m, 0.316mm I.D., 0.25µm coating) with helium (1ml min^{-1}) as a carrier gas using the following temperature programme: 42°C, held for 5 minutes, then rising at 5°C min^{-1} to 150°C and finally rising at 15°C min^{-1} to 270°C; splitless injection used (split close time: 0 minutes, split open time: 4 minutes). All identified fatty acids were identified and confirmed by comparison of spectra and retention times to those of standards (SUPELCO, 37 component FAME mix, Part no. 47885-U, Sigma-Aldrich Company Limited, Gillingham, Dorset, UK).

Total ion count (TIC) was used for each of the peaks on the resultant chromatograms, and these values were normalised to calculate the relative content of each compound in a sample. Additionally, within each group the normalised percentages were averaged to provide a mean percentage contribution.

Statistical data analysis

As in previous chapters, Multi- Dimensional Scaling (MDS) [Primer 5, version 5.2.0] was employed to clarify any underlying trends in the relatedness of compound composition This enabled comparison of spraints from individual male and female European otters and also comparison of 3 different European otter scent mark types (captive spraints, wild spraints and wild anal sac secretion).

The MDS analysis enabled comparison of:

- The spraints from different captive European otters, using:
    - $\log_{10}$ transformation of absolute data
    - presence or absence data

NB this was only carried out for the 'all' fatty acids data set.

- captive spraints, wild spraints and wild anal sac secretion, using:
    - $\log_{10}$ transformation of absolute data
    - presence or absence data

NB this was carried out for 'identified' fatty acids only and also for 'all' fatty acids'.

SIMPER analysis was again applied to the similarity matrices generated for each scent mark group. This examined the contribution of each chemical to the average Bray-Curtis dissimilarity between the sample groups.

Analysis of similarities (ANOSIM) was again carried out using the PRIMER software. This provided a way to test statistically whether there was a significant difference between two or more groups of sampling units (in this case: otter sex groups / otter scent mark groups).

Once again, as in previous chapters, in comparing the spraint profiles, the concept of `overlap' between samples was used.

The differences found between samples were tested for significance using independent *t*-tests. Levene's test for equality of variances was initially employed to identify the most appropriate t-test (equal variances assumed/equal variances not assumed).

RESULTS

Fatty acids extracted

Captive European otter spraint material: GC analysis of captive European otter spraint samples allowed positive identification of 23 fatty acids (see Table 2). Typical spraint chromatographs from 2 otters (1 male and 1 female) are shown in Figure 1. The samples were dominated (dominant acids classified as having a mean % contribution of 2% or more) by five of the identified acids collectively constituting an average of >87% of the mean percentage contribution. These were palmitic, stearic, oleic, linoleic and cis-13,16-docosadienoic. Although these acids on average dominated, their percentage contribution showed some variation between individuals and between samples of individuals (hence the large standard deviation figures observed). The acid with the lowest standard deviation (linoleic) was consistently dominant in every sample. The acid with the largest standard deviation (cis-13,16-docosadienoic) showed much more variation than the others. It was not a dominant acid in every sample and was missing from one sample altogether.

Two possible sulphur based compounds were also identified using the MS library (these were not confirmed with standards). Dimethyl sulphide (eluted at 02:08) which appeared in every sample and dimethyl sulphone (eluted at 03:43) which appeared in 5 of the male samples and 3 of the female samples (see Figure 1).

15 other, unidentified compounds were seen, of which 14 were possible fatty acids and 1 was unknown.[1]

The (dry weight) percentage fatty acid content of the captive European otter spraint samples ranged from 5.21 - 13.05% by mass.

[1] GC/MS software was unable to resolve all chemicals encounted.

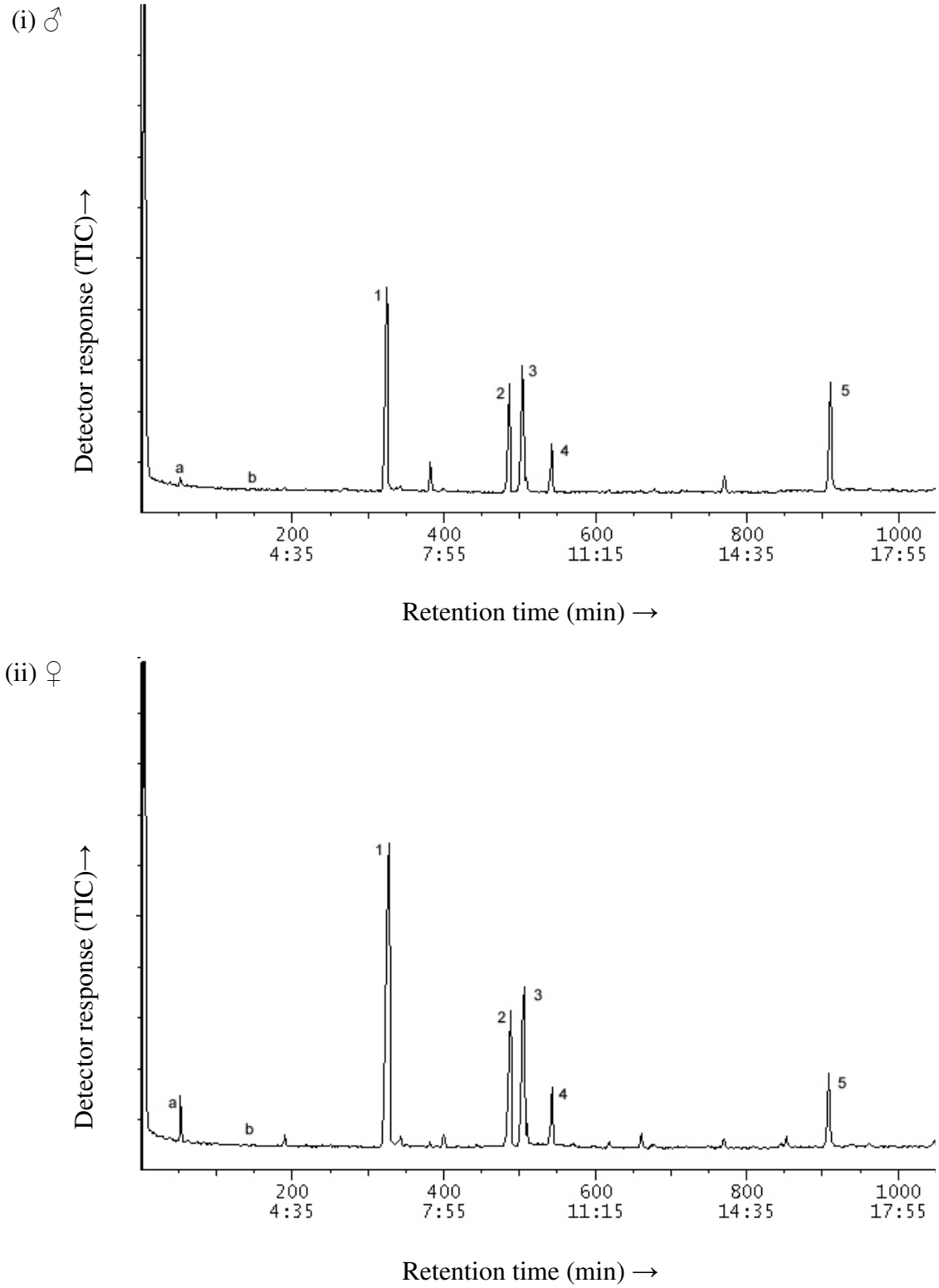


Figure 1. Gas chromatograms of 2 different captive European otter spraint samples (i = male / ii = female). Numbers indicate the 5 dominant Acids (1 = palmitic; 2 = stearic; 3 = oleic; 4 = linoleic; 5 = cis-13,16-docosadienoic). Letters indicate possible sulphur related compounds (a = dimethyl sulphide; b = dimethyl sulphone.

Wild European otter spraint material: GC analysis of wild European otter spraint samples allowed positive identification of 25 fatty acids (see Table 2). Typical spraint chromatographs from 2 wild otters (sex unknown) are shown in Figure 2. The samples were dominated by twelve of the identified acids collectively constituting an average of >91% of the mean percentage contribution. These were myristic, palmitic, palmitoleic, stearic, oleic, elaidic, linoleic, cis-11-eicosenoic, arachidonic, erucic, lignoceric and nervonic. Although these acids on average dominated, their percentage contribution showed some variation between samples (hence the large standard deviation figures observed). For example, erucic acid had a standard deviation that was larger than its mean percentage contribution due to high variation between samples. This variation in erucic acid is illustrated in Figure 2, sample (ii) has a visibly higher amount of erucic acid (compound number 10) compared with sample (i).

Two possible sulphur based compounds were also identified using the MS library (these were not confirmed with standards). Dimethyl sulphide (eluted at 02:07) which appeared in every sample and dimethyl sulphone (eluted at 03:43) which appeared in 4 of the 5 samples examined.

26 other, unidentified compounds were seen, of which 23 were possible fatty acids and 3 were unknown.[1]

The (dry weight) percentage fatty acid content of the wild European otter spraint samples ranged from 0.93 - 2.96% by mass.

[1] GC/MS software was unable to resolve all chemicals encounted.

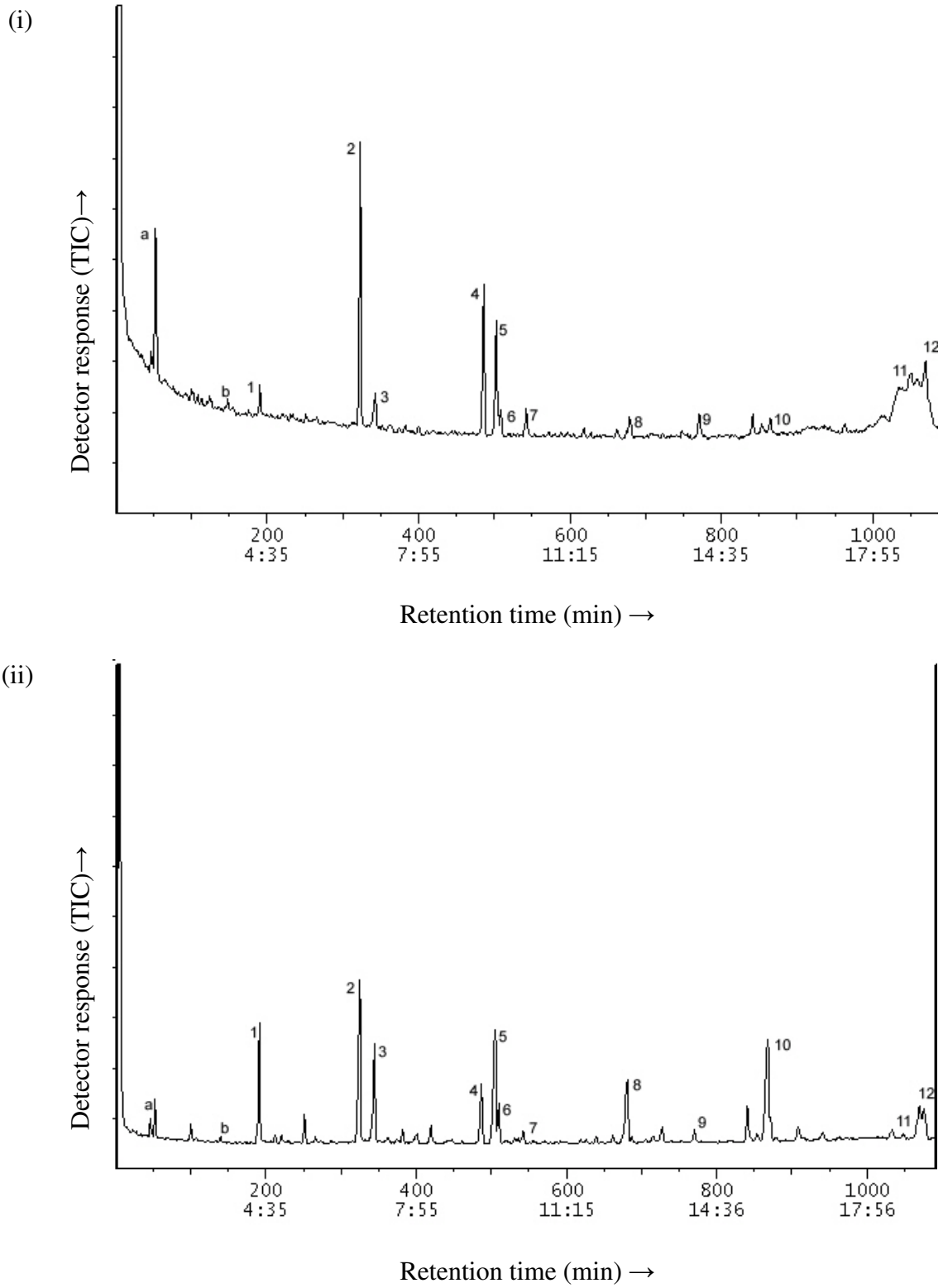


Figure 2. Gas chromatograms of 2 different wild European otter spraint samples (sex unknown). Numbers indicate the 12 dominant Acids (1 = myristic; 2 = palmitic; 3 = palmitoleic; 4 = stearic; 5 = oleic; 6 = elaidic; 7 = linoleic; 8 = cis-11-eicosenoic; 9 = arachidonic; 10 = erucic; 11 = lignoceric; 12 = nervonic). Letters indicate possible sulphur related compounds (a = dimethyl sulphide; b = dimethyl sulphone).

Wild European otter anal sac secretion: GC analysis of wild *L. lutra* anal sac secretion samples allowed positive identification of 22 fatty acids (see Table 2) by comparison to fatty acid standard. Typical spraint chromatographs from 2 wild European otters (1 male / 1 female) are shown in Figure 3. the samples were dominated by thirteen of the identified acids collectively constituting an average of >90% of the mean percentage contribution. these were pentadecanoic, palmitic, palmitoleic, stearic, oleic, elaidic, linoleic, linolelaidic, arachidic, cis-11,14-eicosadienoic, behenic, cis-13,16-docosadienoic and lignoceric. Although these acids on average dominated, their percentage contribution showed variation between individuals (hence the large standard deviation figures observed for some acids). For example, palmitoleic acid had a large standard deviation due to variation between individuals. This variation in palmitoleic acid is illustrated in Figure 3, sample (i) has a visibly higher amount of palmitoleic acid (compound number 3) compared with sample (ii), indeed, there were even examples of fatty acids that were dominant in some samples, but not in all and consequently fell below the 2% cut off point for dominance. For instance, myristic acid which showed a high standard deviation as it was absent from one sample altogether.

No sulphur based compounds were identified in the wild European otter anal sac secretion samples.

37 other, unidentified compounds were also seen, of which 21 were possible fatty acids and 16 were unknown.[1]

The (dry weight) percentage fatty acid content of the wild European otter anal sac secretion samples ranged from 55.12 - 74.52% by mass.

[1] GC/MS software was unable to resolve all chemicals encounted.

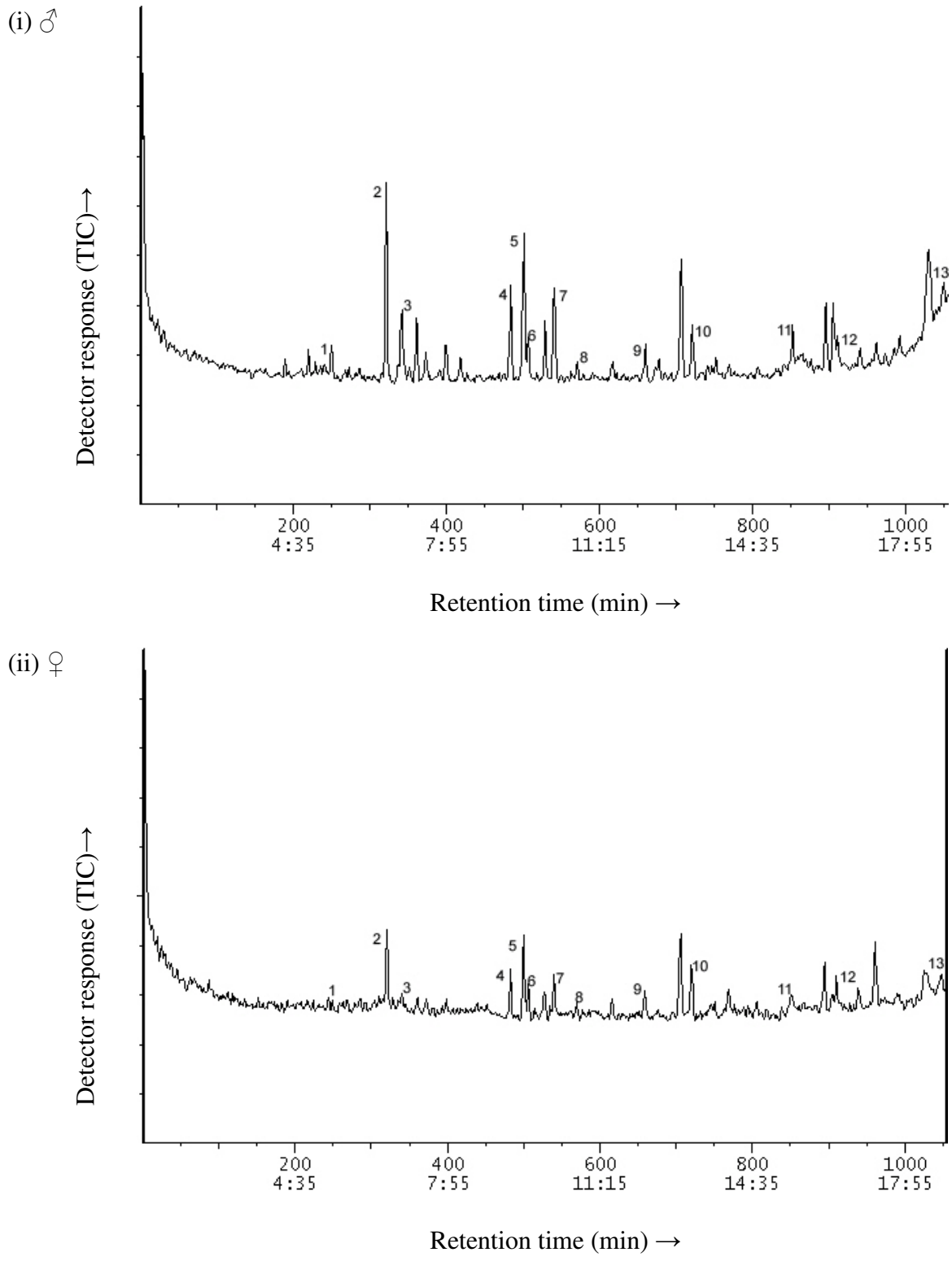


Figure 3. Gas chromatograms of 2 different wild European otter anal sac secretion samples (i = male / ii = female). Numbers indicate the 13 dominant Acids (1 = pentadecanoic; 2 = palmitic; 3 = palmitoleic; 4 = stearic; 5 = oleic; 6 = elaidic; 7 = linoleic; 8 = linolelaidic; 9 = arachidic; 10 = cis-11,14-eicosadienoic; 11 = behenic; 12 = cis-13,16-docosadienoic; 13.= lignoceric). No possible sulphur related compounds were found.

Table 2. Comparison of identified fatty acids present in European otter scent marks: captive otter spraint / wild otter spraint / wild otter anal sac secretion

	Fatty Acid	*C chain*	*Ret. Time (min)*	Captive spraints		Wild spraints		Wild anal sac secretion	
				Mean %	*St dev*	*Mean %*	*St dev*	*Mean %*	*St dev*
1	Capric	C10	02:02			1.3	0.83		
2	Lauric	C12	02:56	0.08	0.11	0.85	0.80	0.07	0.15
3	Tridecanoic	C13	03:35			0.11	0.25		
4	Myristic	C14	04:26	0.79	0.31	3.68†	3.10†	1.46	1.14
5	Myristoleic	C14:1	04:48			0.13	0.30		
6	Pentadecanoic	C15	05:28	0.22	0.16	1.07	0.79	2.92‡	3.00‡
7	cis-10-Pentadecenoic	C15:1	05:48			0.06	0.13		
8	Palmitic	C16	06:40	37.19*	6.37*	20.68†	8.73†	18.25‡	4.38‡
9	Palmitoleic	C16:1	06:59	0.95	0.52	4.88†	3.06†	7.05‡	4.70‡
10	Heptadecanoic	C17	07:58	1.14	0.51	0.77	0.55		
11	cis-10-Heptadecanoic	C17:1	08:19			0.32	0.21		
12	Stearic	C18	09:19	17.17*	2.99*	10.71†	7.08†	7.08‡	1.33‡
13	Oleic	C18:1	09:39	17.20*	4.38*	13.64†	4.66†	12.59‡	1.70‡
14	Elaidic	C18:1	09:42	1.73	0.73	2.07†	1.39†	3.28‡	1.32‡
15	Linoleic	C18:2	10:17	4.92*	0.84*	3.44†	1.89†	7.97‡	1.33‡
16	Linolelaidic	C18:2	10:43	0.29	0.13			2.90‡	0.90‡
17	γ-Linolenic	C18:3	10:48			0.25	0.32		
18	Linolenic	C18:3	11:12	0.20	0.23	0.49	0.42	0.01	0.03
19	Arachidic	C20	12:19	1.32	0.77	0.76	0.52	4.58‡	1.29‡
20	cis-11-Eicosenoic	C20:1	12:38	0.41	0.17	6.79†	4.56†	1.27	0.89
21	cis-11,14-Eicosadienoic	C20:2	13:16	0.23	0.15			9.43‡	3.92‡
22	Cis-8,11,14-Eicosatrienoic	C20:3	13:40	0.25	0.28			1.63	1.06
23	Heneicosanoic	C21	13:46	0.10	0.10			0.52	0.72
24	Arachidonic	C20:4	14:06	1.47	0.54	3.13†	2.02†	1.74	1.62
25	Behenic	C22	15:28	1.14	0.68	0.90	0.48	3.89‡	1.88‡
26	Erucic	C22:1	15:41	0.18	0.19	15.06†	16.29†	0.82	0.80
27	cis-13,16-Docosadienoic	C22:2	16:28	11.38*	12.98*			3.78‡	2.01‡
28	Tricosanoic	C23	17:00	0.67	0.68	1.30	1.70	1.67	1.80
29	Lignoceric	C24	18:43	0.95	0.52	2.94†	3.59†	7.06‡	4.84‡
30	Nervonic	C24:1	19:11			4.67†	5.23†		

***** ***indicates the 5 most dominant acids that together make up >87% of the mean percentage contribution***

† ***indicates the 12 most dominant acids that together make up >91% of the mean percentage contribution***

‡ ***indicates the 13 most dominant acids that together make up >90% of the mean percentage contribution***

indicates fatty acidscommon to all three scent mark groups

indicates fatty acidscommon to one scent mark group only

Scent mark weight and percentage fatty acid content

Captive and wild spraints: comparison of weight (g)

Captive spraint sample weight was significantly higher than that of the wild European otter spraint samples ($t = 5.277$, $df = 12.092$, $p < 0.01$). See Figure 4.

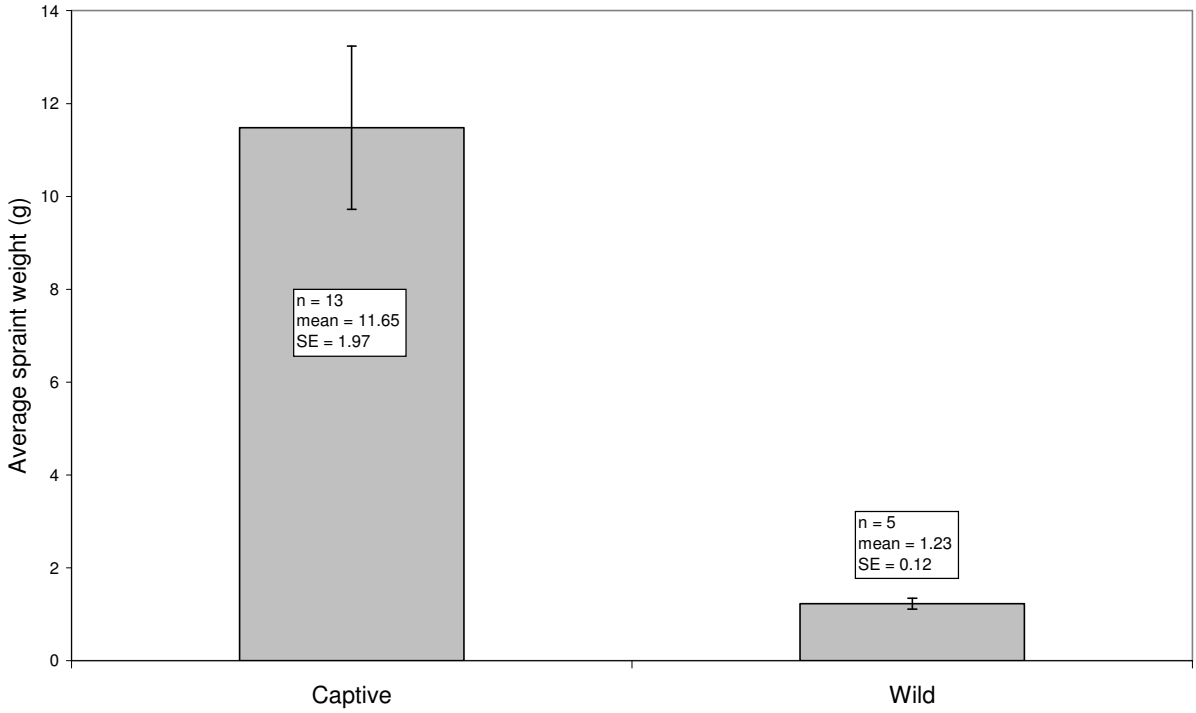


Figure 4. Captive and wild European otter spraints: comparison of weight (g)

Percentage fatty acid content of European otter scent mark material: comparison between captive spraints; wild spraints; wild anal sac secretion

The lowest (dry weight) % fatty acid content was seen in the wild European otter spraint samples. The next highest were the captive spraint samples. The highest % fatty acid content was seen in the wild anal sac secretion samples. See Figure 5.

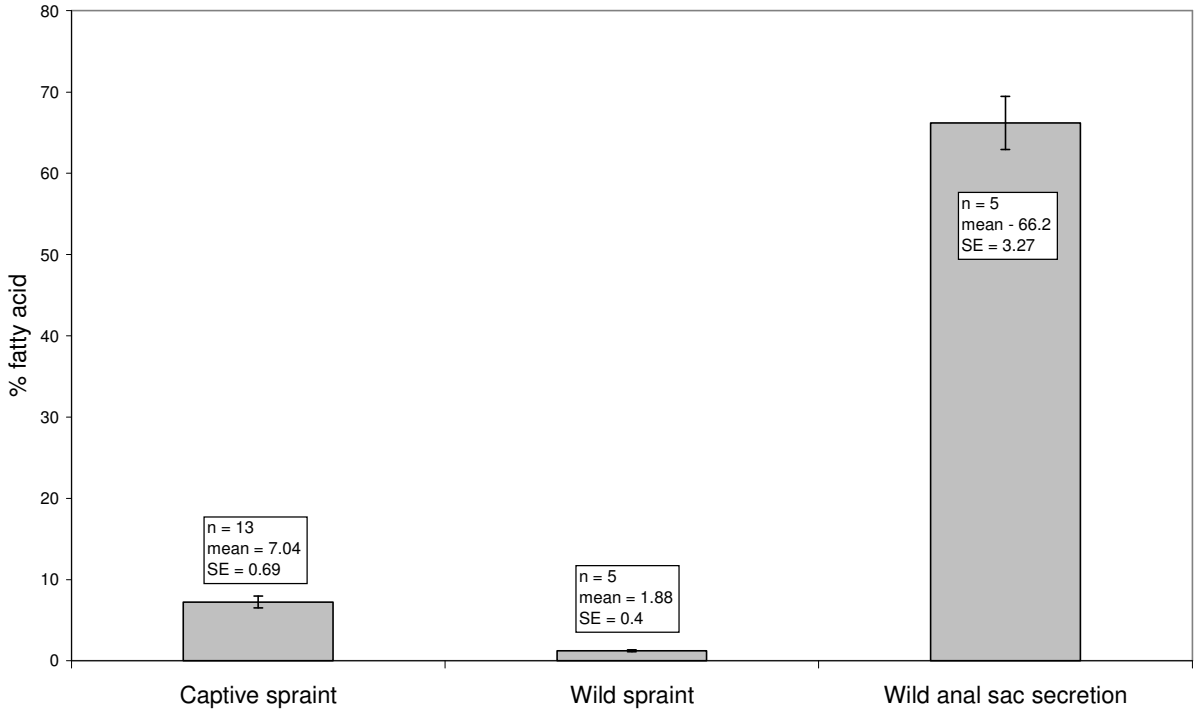


Figure 5. Dry weight % Fatty Acid content of European otter scent mark material: comparison between *captive spraints*; *wild spraints*; *wild anal sac secretion*

All differences were statistically significant. See Table 3.

Table 3. Comparison of % fatty acid content scent mark types – independent *t*-test results

Comparison	*t value*	*df*	*Sig.(2-tailed)*
Captive spraints vs *Wild spraints*	4.434	16	<0.001
Captive spraints vs *Wild anal sac secretion*	17.717	4.365	<0.001
Wild spraints vs *Wild analsac secretion*	19.542	4.120	<0.001

Comparison of identified fatty acids present in captive otter spraint / wild otter spraint / wild otter anal sac secretion

17 of the identified fatty acids were found to be common to all three groups (see Table 2). There were also 7 fatty acids that were unique to one group only (wild *spraints*).

Overlap in fatty acid composition within and between the sex groups in captive European otter spraints

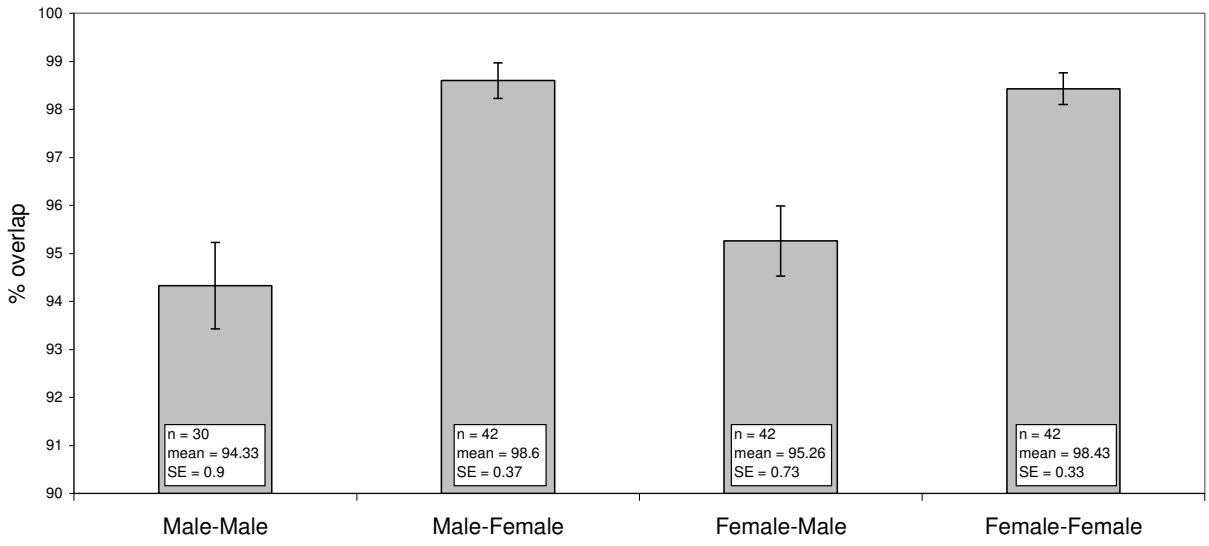


Figure 6. Difference in compound overlap within and between the captive European otter sex groups.

Figure 6 shows the difference in fatty acid overlap between the sexes. The mean overlap within the male group was lower than the mean overlap between males and females. In contrast the mean overlap within the female group was higher than the mean overlap between females and males. The levels of significance of these differences were calculated using an independent *t*-test. All differences were statistically significant. See Table 4.

Table 4. Comparison of overlap in fatty acid composition within and between European otter sex groups – independent t-test results.

Comparison	*t value*	*df*	*Sig.(2-tailed)*
Male-Male vs *Male-Female*	-4.495	38.512	<0.001
Female-Female vs *Female-Male*	3.978	57.209	<0.001

Overlap in fatty acid profile within and between individuals

Figure 7 shows the mean overlap in the fatty acid profile between different samples from the same individual (`intra-individual overlap') compared with the overlap of each individual with the other individuals of the same sex (`inter-individual overlap').

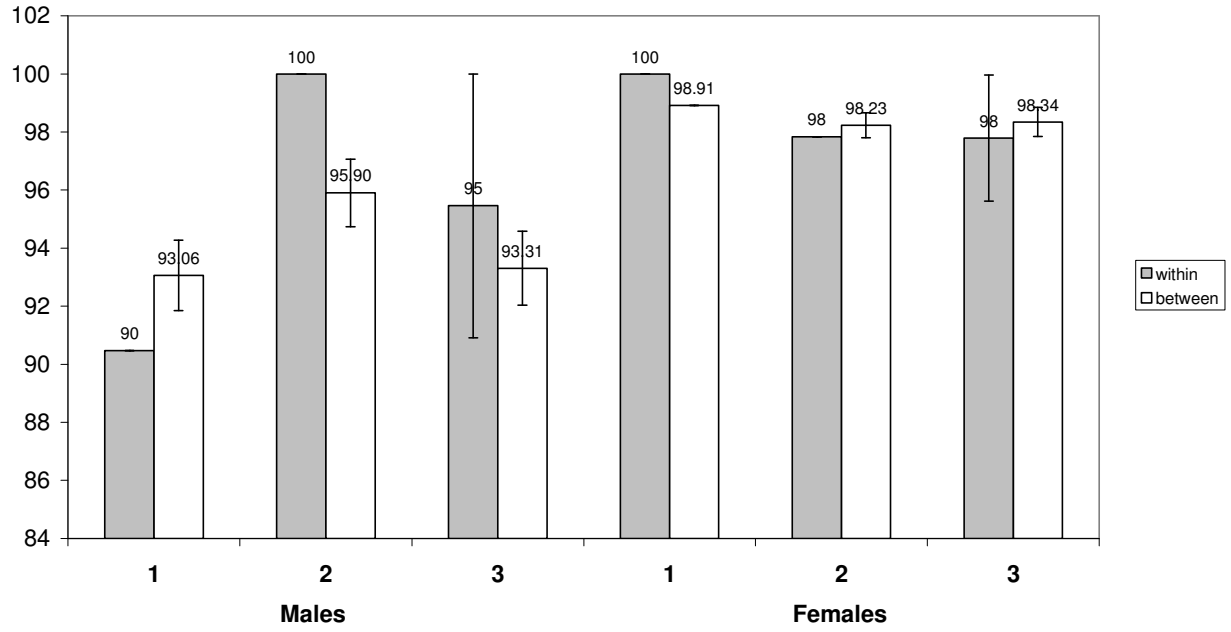


Figure 7. Percentage overlap in fatty acid within and between individuals of the same sex groups. males are shown to the left and females to the right.

Two of the three males had a higher intra-individual overlap than inter-individual overlap. The remaining male had a higher inter-individual overlap than intra-individual overlap.

One of the three females had a higher intra-individual overlap than inter-individual overlap. The remaining females had a higher inter-individual overlap than intra-individual overlap.

No statistical tests were used to analyse these data for significant differences due to the low number of intra-individual samples.

Differences in absolute and relative content of common compounds between the sexes

Table 3 shows five dominant fatty acids were found in the profile of captive European otter spraint material (palmitic, stearic, oleic, linoleic and cis-13,16-docosadienoic). The absolute content (TIC) of each of these fatty acids was measured as the area underneath the peak as seen on their respective chromatograms. The absolute content of each of the five dominant fatty acids for males and females are depicted in Figure 7.

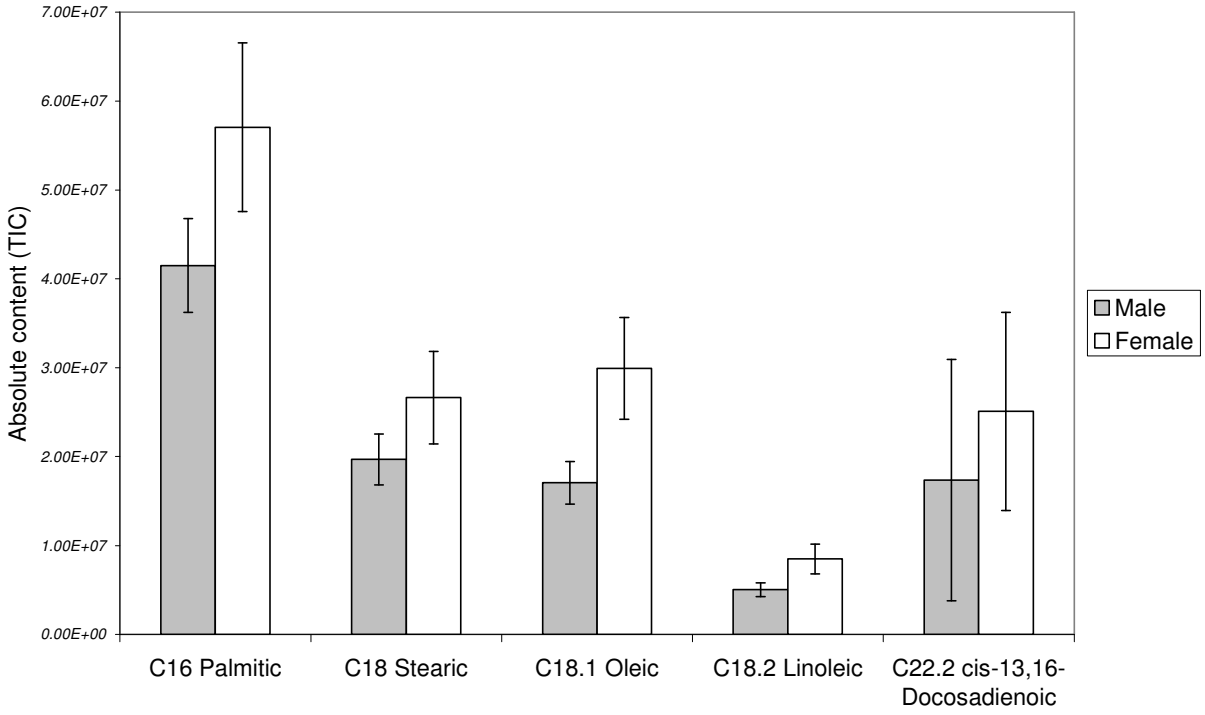


Figure 8. Sex difference in 'absolute' content for dominant Fatty Acids in captive European otter otter spraints

Females appear to have a higher absolute content of each of the 5 dominant fatty acids than their male counterparts, however, none of these differences were found to be significant. The results for oleic ($t = -2.07$, $df = 7.987$, $p = 0.072$) and linoleic ($t = -1.752$, $df = 11$, $p = 0.108$) were closer to being significantly different than the other acids

The relative content (%) of each of the five dominant fatty acids was also calculated for males and females. The relative content of each of the five dominant fatty acids for males and females are depicted in Figure 8.

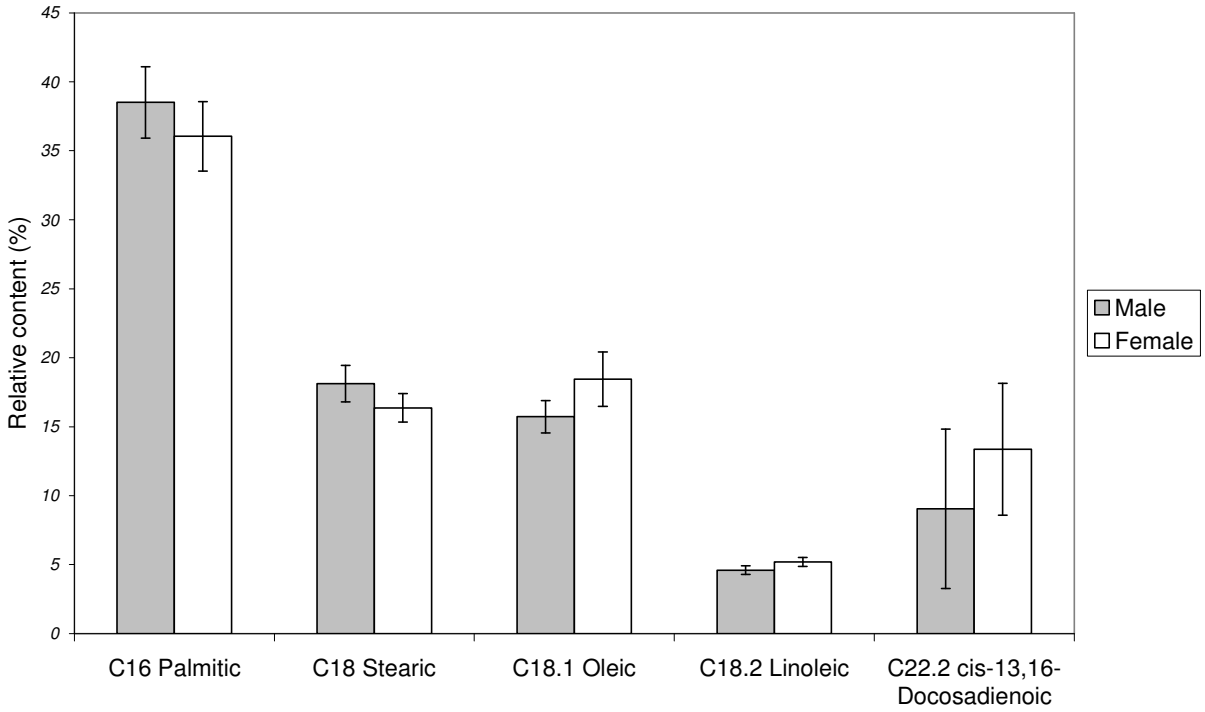


Figure 9. Sex difference in 'relative' content for dominant Fatty Acids in captive European otter spraints

Females appear to have a higher relative content of three of the five dominant fatty acids than their male counterparts (oleic, linoleic, cis-13,16-docosadienoic). Males had a higher relative content in the remaining two fatty acids (palmitic and stearic). Again, no significant difference in content was seen between the sexes for any of the five dominant fatty acids.

Difference in compound overlap within and between European otter spraint and anal sac secretion groups

Identified fatty acids

Table 5 shows the difference in 'identified' fatty acid overlap between European otter spraint (captive / wild) and anal sac secretion groups.

Table 5. Comparison of 'identified fatty acid' % overlap within and between European otter scent mark groups.

		captive spraints	*wild spraints*	*wild ASS*
captive spraints	***compound overlap %***	**96.78**	**78.06**	**82.99**
	n	156	65	65
	SE	0.33	0.38	0.94
wild spraints	***compound overlap %***	**77.29**	**94.89**	**65.52**
	n	65	20	25
	SE	1.29	1.24	1.47
wild ASS	***compound overlap %***	**95.22**	**75.57**	**93.03**
	n	65	25	20
	SE	1.32	0.6	1.66

The levels of significance of the differences between the different scent mark groups were calculated using independent *t*-tests. Initially, Levene's test for equality of variances was carried out. This determined the type of *t*-test subsequently used (i.e., assuming equal variances or assuming unequal variances). See Table 6 for results.

Table 6. *t* –test results showing the levels of significance of the differences between the different European otter scent mark groups for identified fatty acids.

		captive spraints vs captive spraints	*wild spraints vs wild spraints*	*wild ASS vs wild ASS*
captive spraints vs wild spraints	*t* value	37.111	7.263	-
	df	159.407	83	-
	Sig.(2-tailed)	***<0.001***	***<0.001***	-
captive spraints vs wild ASS	*t* value	13.768	-	-0.862
	df	80.184	-	83
	Sig.(2-tailed)	***<0.001***	-	*0.391*
wild spraints vs wild ASS	*t* value	-	15.223	9.905
	df	-	42.881	23.931
	Sig.(2-tailed)	-	***<0.001***	***<0.001***

NB - p values shown in italics, significant results also in bold.

All fatty acids

Table 7 shows the difference in 'total compound' overlap between European otter spraints (captive / wild) and anal sac secretion groups.

Table 7. Comparison of 'all fatty acid' % overlap within and between European otter scent mark groups

		captive spraints	*wild spraints*	*wild ASS*
captive spraints	***compound overlap %***	**94.8**	**80.1**	**72.46**
	n	156	65	65
	SE	0.31	0.61	0.94
wild spraints	***compound overlap %***	**69.71**	**88.04**	**61.68**
	n	65	20	25
	SE	0.93	2.01	1.77
wild ASS	***compound overlap %***	**51.01**	**49.71**	**90.45**
	n	65	25	20
	SE	0.45	1.06	1.62

The levels of significance of the differences between the different scent mark groups were calculated using independent *t*-tests. Initially, Levene's test for equality of variances was carried out. This determined the type of *t*-test subsequently used (i.e., assuming equal variances or assuming unequal variances). See Table 8 for results.

Table 8. *t*-test results showing the levels of significance of the differences between the different European otter scent mark groups for *all* fatty acids.

		captive spraints vs captive spraints	*wild spraints vs wild spraints*	*wild ASS vs wild ASS*
captive spraints vs wild spraints	*t* value	21.818	9.130	-
	df	97.638	83	-
	p value	***<0.001***	***<0.001***	-
captive spraints vs wild ASS	*t* value	30.560	-	23.484
	df	91.633	-	21.981
	p value	***<0.001***	-	***<0.001***
wild spraints vs wild ASS	*t* value	-	9.873	21.074
	df	-	43	33.802
	p value	-	***<0.001***	***<0.001***

NB – significant p values shown in bold and italic.

Comparison of spraints from captive European otter individuals using Multi-Dimensional Scaling (MDS)

***All fatty acids* – absolute content data, log_{10} transformed** 1 way ANOSIM [R=0.11, p=0.103]

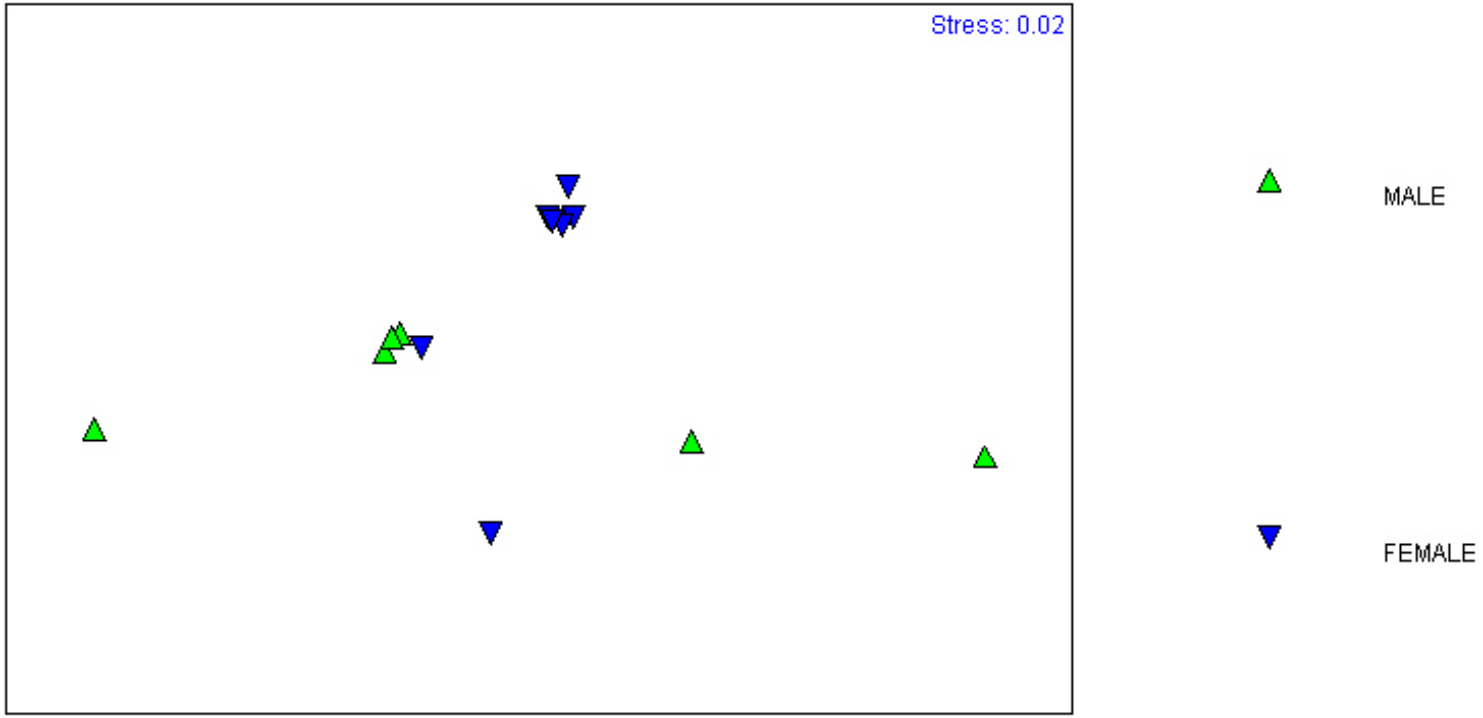


Figure 10. Multi Dimensional Scaling diagram showing relatedness between individual European otter spraints, based on log_{10} transformed absolute content data (using 'all' fatty acid data). ANOSIM R value indicates no significant differences between the sexes.

***All fatty acids* – presence or absence data** 1 way ANOSIM [R=0.324, p=0.005]

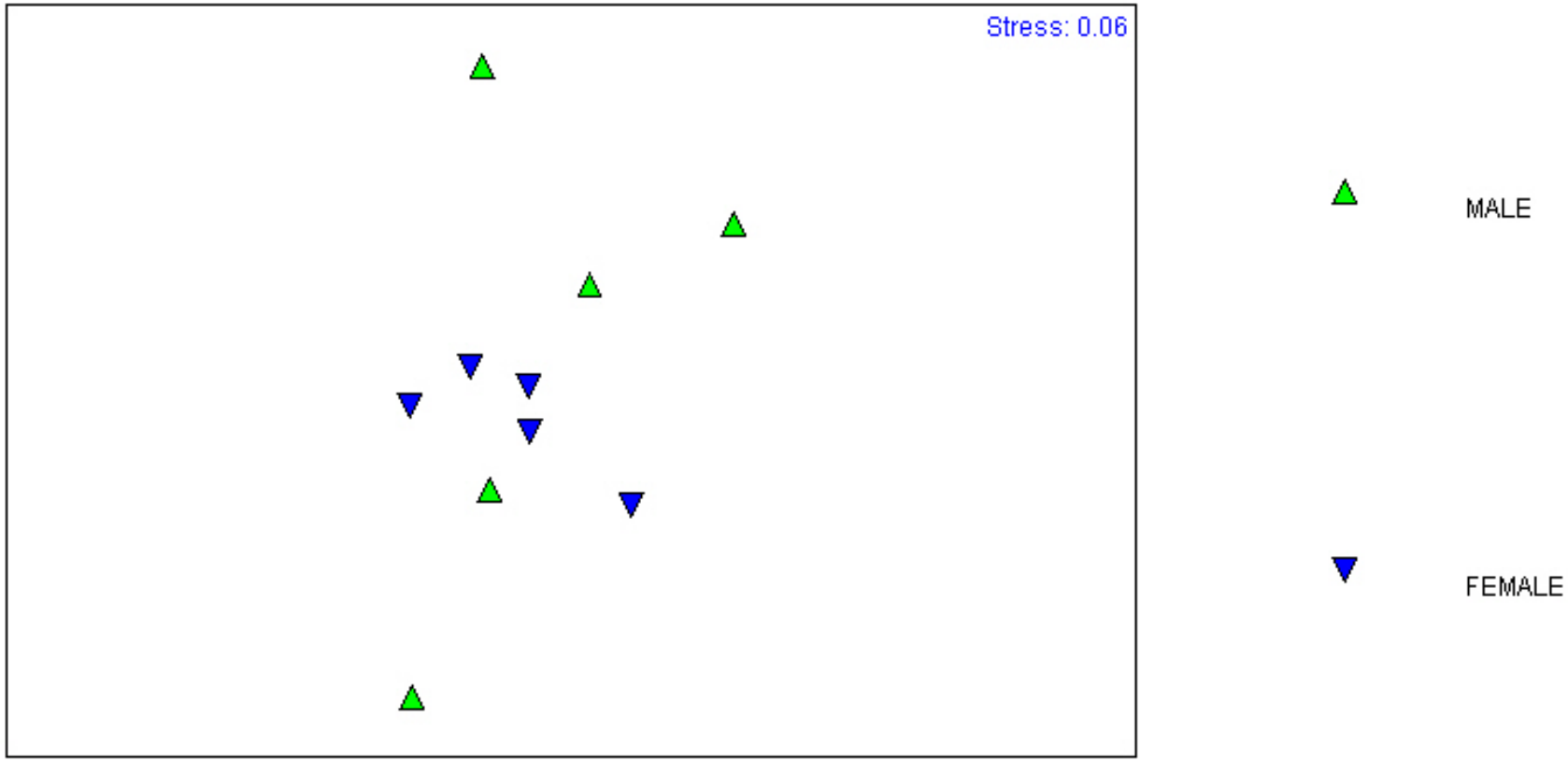


Figure 11. Multi Dimensional Scaling diagram showing relatedness between individual European otter spraints, based on presence or absence data (using all Fatty Acid data). ANOSIM R value indicates no significant differences between the sexes.

The MDS analysis results for the absolute data showed the possibility of separation based on sex. The presence or absence data showed a slightly different result to the absolute content data with all the female samples grouped together within a much wider spread of male samples. However, ANOSIM R values indicated no significant difference between the sexes for the absolute data whilst a weak significant difference between sexes was recorded for the presence absence data.

Analysis was now carried out in order to ascertain which chemicals might be most responsible for the possible sexual separation observed.

SIMPER analysis

Similarity / dissimilarity summary tables

Comparison of spraints from captive male and female European otter individuals

Table 9. Average percentage similarity (plus fatty acids with the largest contribution to similarity) within sex groups.

Data type	Male	Female
	Average % Similarity	
absolute content (log_{10} transformed)	85.43	92.68
presence or absence	92.80	97.72
	Fatty acid (average % contribution to similarity)	
absolute content (log_{10} transformed)	palmitoleic (13.48)	fatty acid 41 (12.27)
presence or absence	arachidic (3.2)	arachidic (2.87

Table 10. Average percentage dissimilarity (plus fatty acids with the largest contribution to dissimilarity) between sex groups.

Data type	Male v Female	
	Average % dissimilarity	*Fatty acid (average % contribution to dissimilarity)*
absolute content (log_{10} transformed)	11.45	fatty acid 4 (24.4)
presence or absence	5.46	fatty acid 45 (17.57)

The presence or absence data has a single dominant acid in both males and females (arachidic). For the absolute data the dominant acid differs between males and females (males – palmitoleic; females – fatty acid 41).

Comparison of European otter spraints (wild and captive) and anal sac secretion (wild) using Multi-Dimensional Scaling (MDS)

***Identified fatty acids* - absolute content data, log_{10} transformed** 1 way Global ANOSIM [R=0.93, p=0.001]

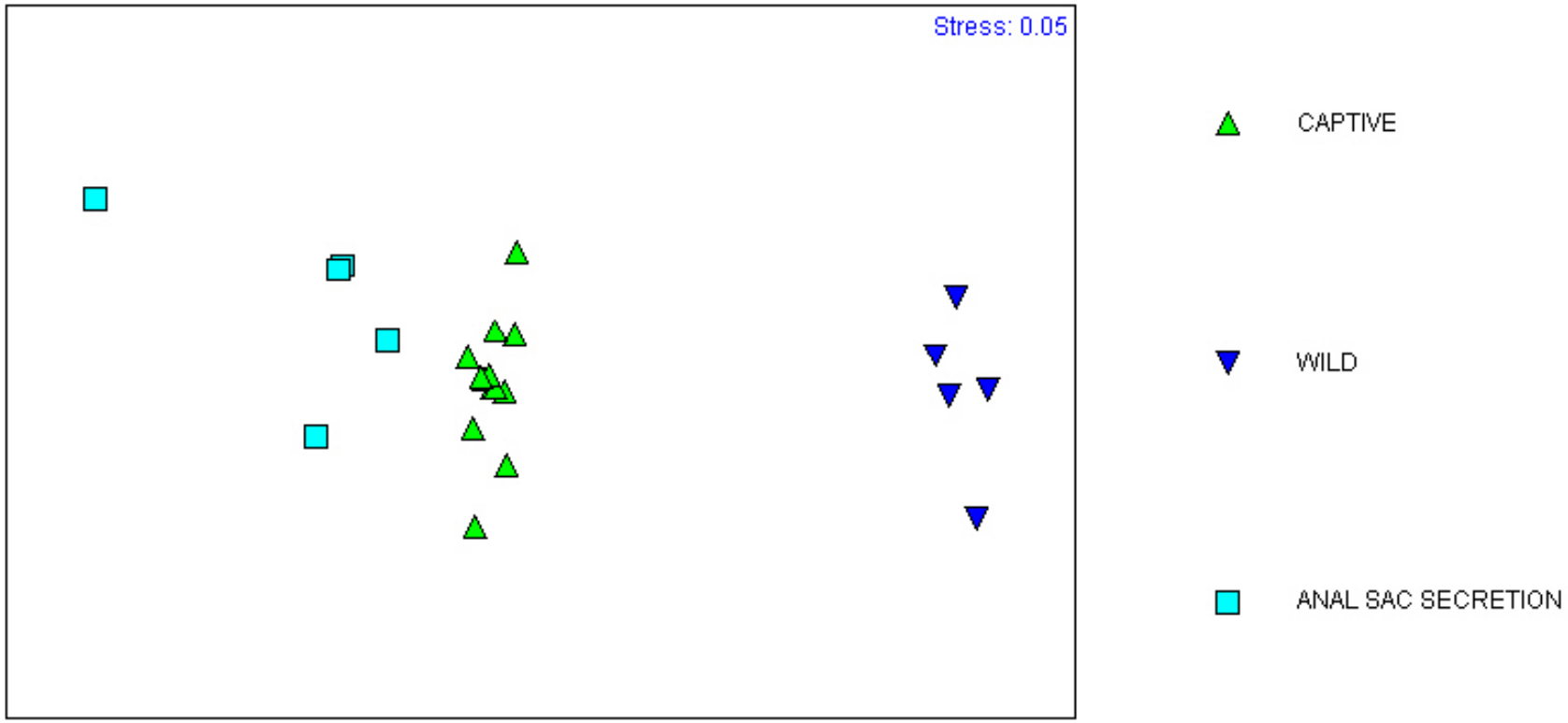


Figure 12. Multi Dimensional Scaling diagram showing relatedness between captive spraints, wild spraints and wild anal sac secretion, based on log_{10} transformed absolute content data (using only 'identified' fatty acid data). Global ANOSIM R value indicates an overall significant difference between the scent mark types.

***Identified fatty acids* – presence or absence data** 1 way Global ANOSIM [R=0.83, p=0.001]

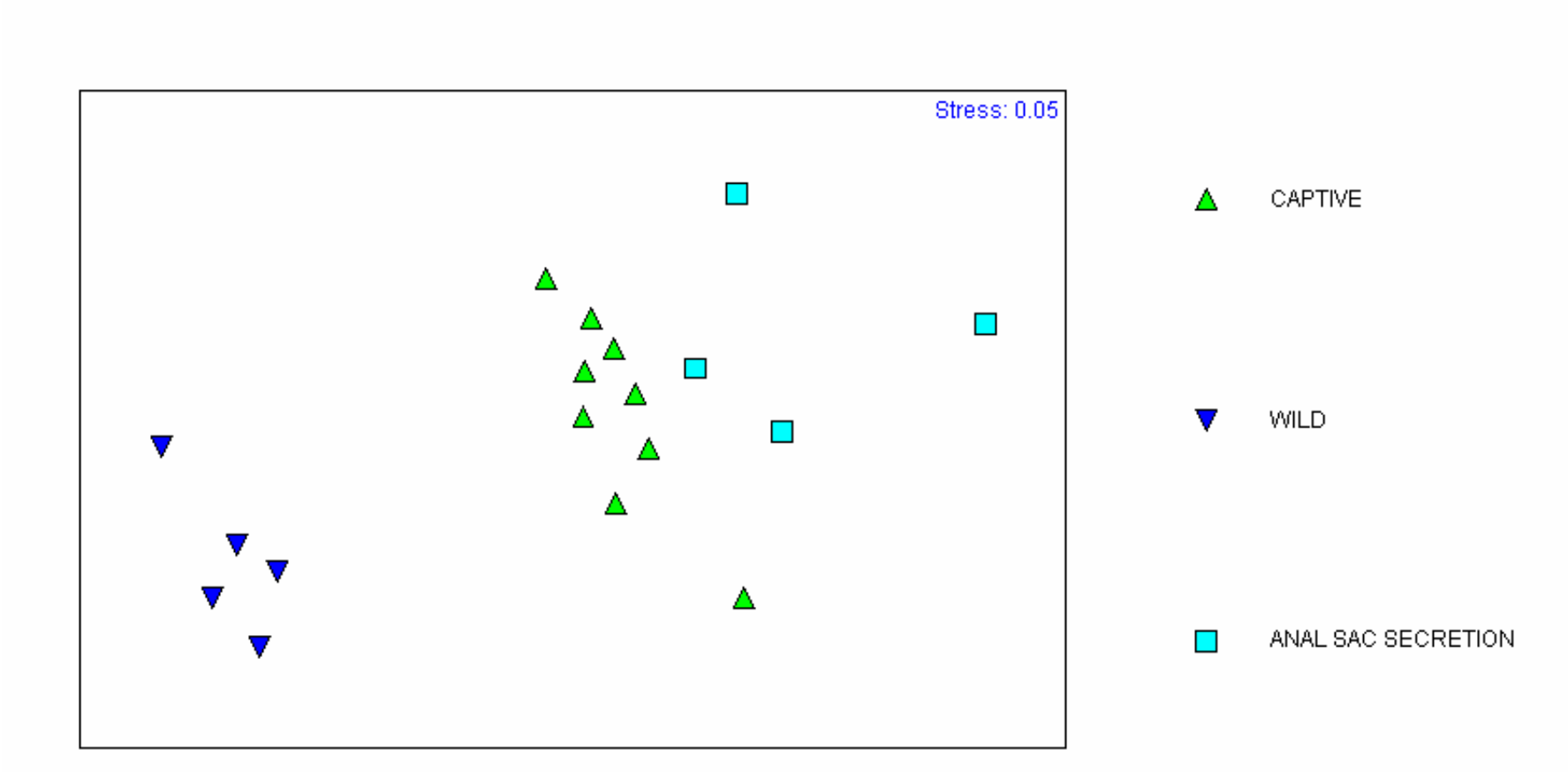


Figure 13. Multi Dimensional Scaling diagram showing relatedness between captive spraints, wild spraints and wild anal sac secretion, based on presence or absence data (using only 'identified' fatty acid data). Pairwise Global ANOSIM R value indicates an overall significant difference between the scent mark types.

***All fatty acids* - absolute content data, log_{10} transformed** 1 way Global ANOSIM [R=0.99, p=0.001]

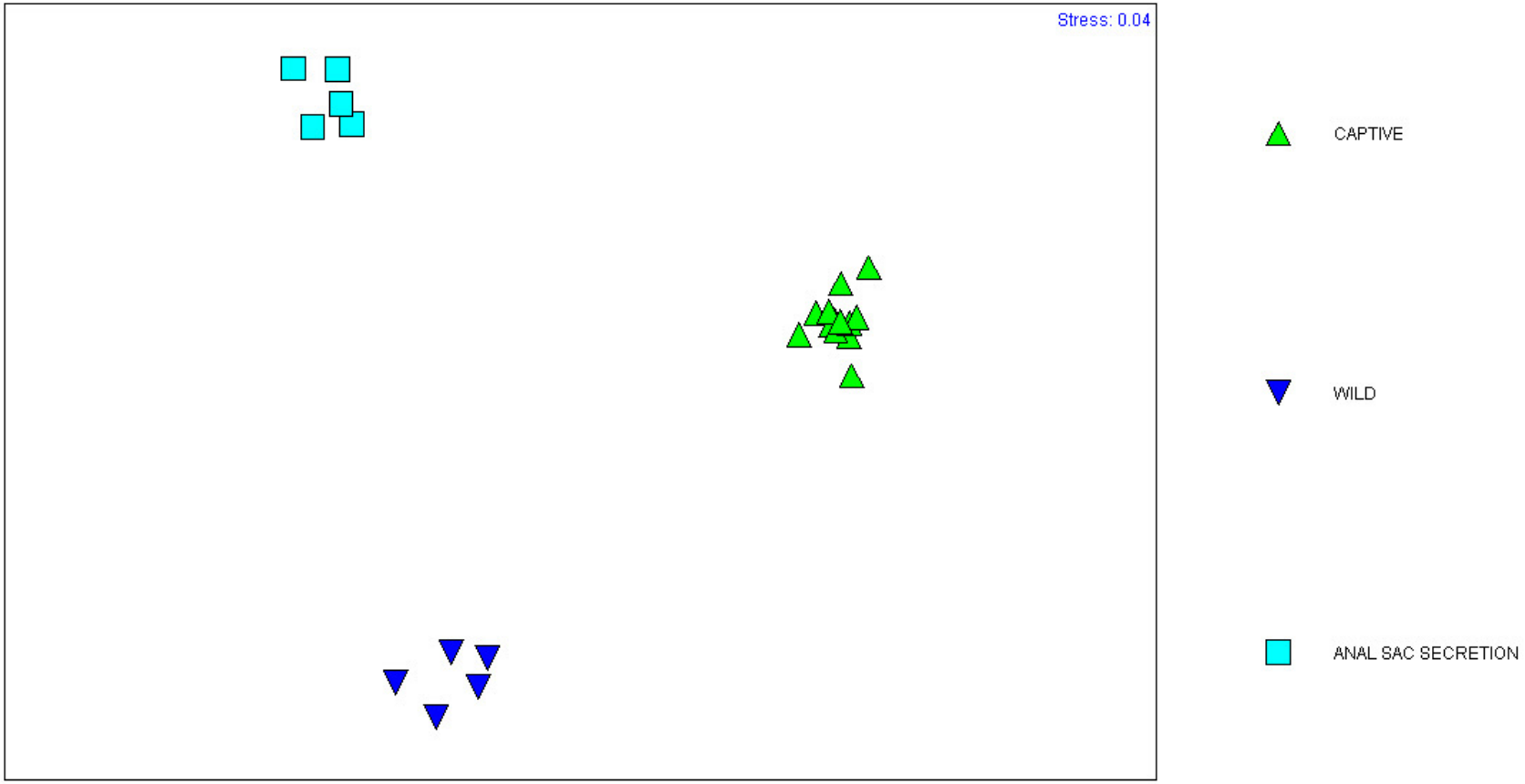


Figure 14. Multi Dimensional Scaling diagram showing relatedness between captive spraints, wild spraints and wild anal sac secretion, based on log_{10} transformed absolute fatty acid data (using all fatty acids extracted). Global ANOSIM R value indicates an overall significant difference between the scent mark types.

***All fatty acids* – presence or absence data** 1 way Global ANOSIM [R=0.99, p=0.001]

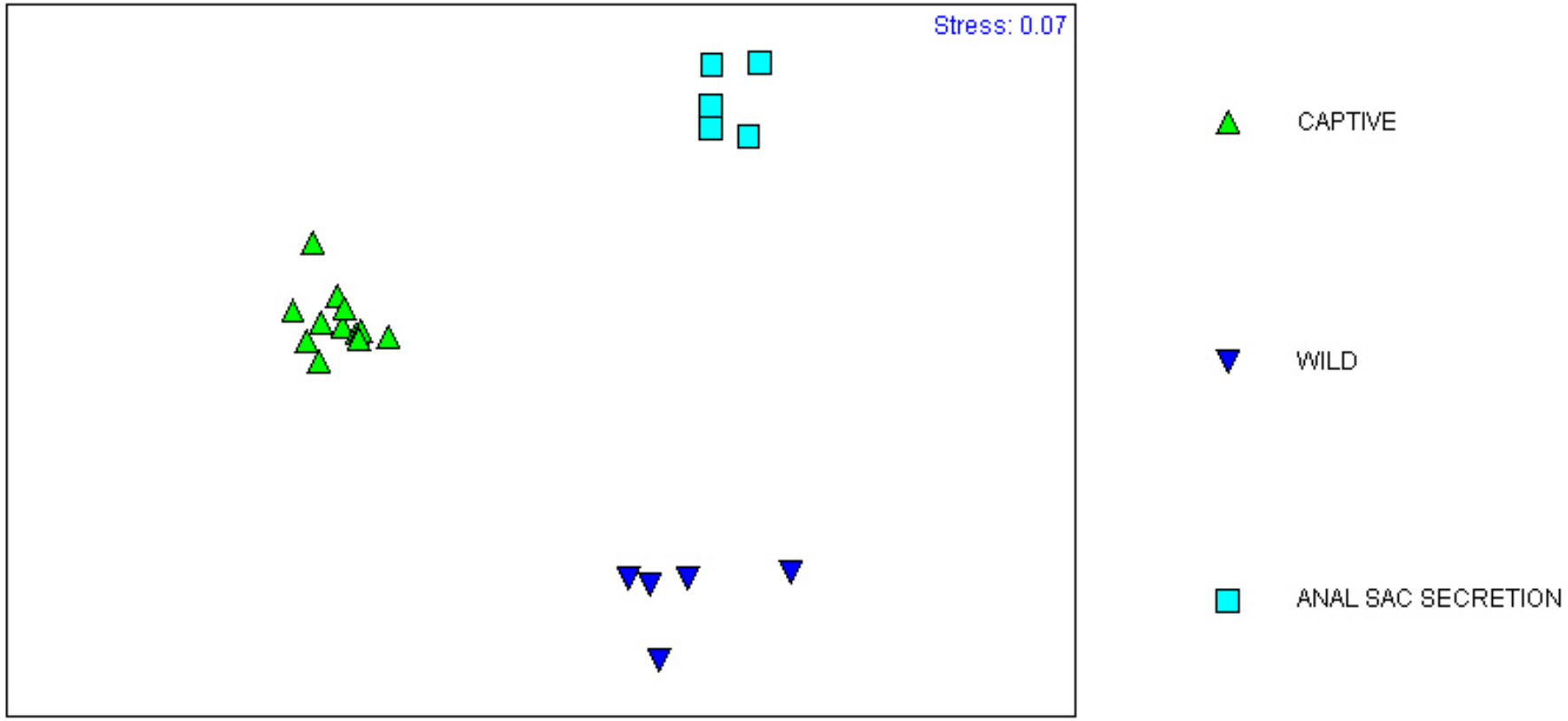


Figure 15. Multi Dimensional Scaling diagram showing relatedness between captive spraints, wild spraints and wild anal sac secretion, based on presence or absence Fatty Acid data (using all Fatty Acids extracted). Global ANOSIM R value indicates an overall significant difference between the scent mark types.

The MDS analysis for the identified fatty acids showed a good measure of separation between the 3 otter scent mark groups for the absolute data. The MDS analysis results for the presence or absence data showed a slightly less definite than the result seen for the absolute content data. The MDS analysis for all fatty acids showed a definite separation between the 3 otter scent mark groups. This was a much better split that was seen using the 'identified' fatty acids alone. The MDS analysis for all fatty acids, in general, showed much more definite separation into the 3 otter scent mark groups than when only the 'identified' fatty acids were used.

These results were confirmed by the Global ANOSIM results for both data types (absolute and presence or absence) and for both the identified and all fatty acids analyses. A significant overall difference between scent mark types was seen. Pairwise comparisons between separate pairs of scent mark types were also made. Table 11 shows the results of these comparisons for both data types.

Table 11. Pairwise Anosim comparison of otter scent mark types (*Abs* – Absolute data; *P/A* – Presence or Absence data)

	Groups	R statistic		p value		Possible Permutations	Actual Permutations
		Abs	*P/A*	*Abs*	*P/A*	(for both data types)	(for both data types)
	Identified fatty acids						
1	captive, wild	1	0.999	0.001	0.001	8568	999
2	captive, anal sac secretion	0.856	0.593	0.008	0.001	8568	999
3	wild, anal sac secretion	1	1	0.001	0.008	126	126
	All fatty acids						
4	captive, wild	0.998	0.996	0.001	0.001	8568	999
5	captive, anal sac secretion	1	1	0.001	0.001	8568	999
6	wild, anal sac secretion	1	1	0.008	0.008	126	126

All pairwise comparisons indicate a significant difference between each pair of scent mark types with the majority of R values equal to or approaching 1.

Analysis was now carried out in order to ascertain which chemicals might be most responsible for the separation observed.

SIMPER analysis

Similarity / dissimilarity summary tables

Comparison of European otter spraints (wild and captive) and anal sac secretion (wild)

Table 12 summarises the average percentage similarities within the otter scent mark groups.

Table 12. Summary of average percentage similarity within the scent mark groups in terms of Fatty Acid profile.

Data type		Average % Similarity		
		captive spraints	wild spraints	anal sac secretion
Identified Fatty Acids	*absolute content* data, log_{10} transformed	93.38	90.26	90.18
	presence or absence data	91.86	89.88	86.92
All Fatty Acids	*absolute content* data, log_{10} transformed	91.15	83.73	87.14
	presence or absence data	92.53	86.42	89.51

Table 13 summarises the average percentage dissimilarities within the otter scent mark types.

Table 13. Average percentage dissimilarity between scent mark groups

Data type		Average % Dissimilarity		
		captive v wild	captive v anal sac secretion	wild v anal sac secretion
Identified Fatty Acids	*absolute content* data, log_{10} transformed	24.33	14.63	32.55
	presence or absence data	25.47	16.32	34.89
All Fatty Acids	*absolute content* data, log_{10} transformed	28.72	30.32	36.15
	presence or absence data	26.30	26.07	32.78

Table 14 summarises the dominant fatty acids responsible for similarities within scent mark groups (both for the 'identified' fatty acid data and the 'all' fatty acid data).

Table 14. Summary of fatty acids with the largest contribution to similarity within the scent mark groups

Data type		Fatty Acid (average % contribution to similarity)		
		captive spraints	wild spraints	anal sac secretion
Identified Fatty Acids only	*absolute content* data, $\log_{10}$ transformed	palmitic (6.11)	palmitic (5.86)	palmitic (6.51)
	presence or absence data	arachidic (4.88)	arachidic (4.89)	linolelaidic (5.82)
All Fatty Acids	*absolute content* data, $\log_{10}$ transformed	palmitic (4.11)	palmitic (3.7)	palmitic (3.39)
	presence or absence data	fatty acid 52 (3.22)	lignoceric (3.07)	fatty acid 46 (2.99)

The presence or absence data seemed to have a varied contributor to percentage similarity within the groups (NB this may be explained by the top ten contributors here having mostly identical amounts of percentage contribution due to the binary nature of the data).

Table 15 summarises the fatty acids that have the largest contribution to dissimilarity between the scent mark groups for each data type.

Table 15. Summary of fatty acids with the largest contribution to dissimilarity between the scent mark groups

Data type		Fatty Acid (average % contribution to dissimilarity)		
		captive v wild	captive v anal sac secretion	wild v anal sac secretion
Identified fatty acids only	*absolute content* data, log_{10} transformed	cis-13,16-docosadienoic (9.82)	heptadecanoic (17.33)	cis-11,14-eicosadienoic (7.9)
	presence or absence data	cis-11,14-eicosadienoic (9.12)	heptadecanoic (14.23)	linolelaidic (7.68)
All fatty acids	*absolute content* data, log_{10} transformed	fatty acid 27 (5.1)	fatty acid 42 (5.17)	fatty acid 42 (4.14)
	presence or absence data	fatty acid 52 (5.34)	fatty acid 46 (5.41)	fatty acid 46 (4.07)

Here, the dissimilarity between groups is not dominated by a specific fatty acid (as seen with the similarity within groups): instead, there are a variety of acids responsible.

DISCUSSION

Fatty acids extracted

Analysis of captive European otter spraints revealed the presence of fatty acids in agreement with previous work on deposited otter scent marks (Gorman et al, 1978). This study has gone further and 'identified' individual fatty acids in otter scent marks. Three groups were examined:

- **captive European otter spraints** (23 fatty acids identified in the range C_{12}-C_{24})
- **wild European otter spraints** (25 fatty acids identified in the range C_{10}-$C_{24.1}$)
- **wild European otter anal sac secretion** (22 fatty acids were identified in the range C_{12}-C_{24})

All those identified were high molecular weight long-chain acids (C_{10}-$C_{24.1}$) of low volatility. Although volatile fatty acids were not detected, molecular weights ranged from 172.3 – 366.62 with 18 of the total 30 'identified' fatty acids (C_{10}-$C_{18.3}$) falling below a molecular weight of 300 – the upper limit for airborne pheromones (Bradbury & Vehrencamp 1998).

Other, unidentified fatty acids were also extracted:

- 14 in the captive European otter spraints
- 23 in the wild European otter spraints
- 21 in the wild European otter anal sac secretion

Previous studies on the anal sac secretions of other mustelids have revealed the presence of fatty acids in only two other species. In mink (*Mustela vison*) anal sac secretion all fatty acids detected were volatile in nature and none were individually identified (Sokolov et al, 1980).

However, the anal gland secretion of the European badger (*Meles meles*) has been shown to contain only long chain, high molecular weight acids in a similar range (C_{14}-C_{24}) to that found in the otter scent mark material (C_{10}-$C_{24.1}$) (Davies et al, 1988). Eleven of the

fifteen acids identified in badger anal sac secretion matched those found in captive otter spraint material. Twelve of the fifteen acids identified in badger anal sac secretion matched those found in wild European otter spraint material. And twelve of the fifteen acids identified in badger anal sac secretion matched those found in wild European otter anal sac secretion material.

In the badger samples between 44% and 52% of the total concentration of fatty acids was formed by five acids (palmitic, stearic, oleic, linoleic and eicosatrienoic). In the captive European otter spraint samples, on average >87% of the total concentration of fatty acids was also dominated by just five acids (*palmitic, *stearic, *oleic, *linoleic and cis-13,16-docosadienoic). *4 fatty acids matched those found in badger. In the wild European otter spraint samples, on average >91% of the total concentration of fatty acids was dominated by twelve acids. And in the wild European otter anal sac secretion samples, on average >90% of the total concentration of fatty acids was dominated by thirteen acids (see Table 16).

Table 16. Comparison between badger and European otter scent mark fatty acids

	European badger (Davies et al, 1988)	European otter		
	Wild anal sac secretion	Captive spraints	Wild spraints	Wild anal sac secretion
No. of Fatty Acids identified	15	23	25	22
Fatty Acid range	C_{14}-C_{24}	C_{12}-C_{24}	C_{10}-$C_{24.1}$	C_{12}-C_{24}
Dominant Fatty Acids	1. palmitic 2. stearic 3. oleic 4. linoleic 5. eicosatrienoic	1. palmitic 2. stearic 3. oleic 4. linoleic 5. cis-13,16-docosadienoic	1. myristic 2. palmitic 3. palmitoleic 4. stearic 5. oleic 6. elaidic 7. linoleic 8. cis-11-eicosenoic 9. arachidonic 10. erucic 11. lignoceric 12. nervonic	1. pentadecanoic 2. palmitic 3. palmitoleic 4. stearic 5. oleic 6. elaidic 7. linoleic 8. linolelaidic 9. arachidic 10. cis-11,14-eicosadienoic 11. behenic 12. cis-13,16-docosadienoic 13. lignoceric
Dominant Fatty Acid percentage contribution	44-52%	87%	91%	90%

Davies et al (1988) suggested that badger anal sac secretion, due to the presence of long chain fatty acids of low volatility, would be well suited to long term signalling and may function in badger territoriality. This could also be true of the fatty acid content of the otter scent mark samples. Otters, however, mark their territory differently to other

carnivorous mammals, e.g., badger and the spotted hyaena, *Crocuta crocuta* where boundary scent marking is used (Kruuk, 1972, 1978b, 1989; Mills, 1990). Instead of marking all the boundaries of their territories, European otters limit their scent marks to particular areas within them (Kruuk, 1992). Kruuk (1992) investigated a possible link between sprainting behaviour and territoriality. Sprainting rates along four known territorial borders were recorded and compared with sprainting rates elsewhere in the territory - the differences were found not to be significant. The otter is more specialized than many other mustelid species and is restricted to particular types of habitat, which has an effect on their territorial organization. The linear ranges of European otter living alongside both freshwater rivers and coastline results in a territory that is less defensible economically than, e.g., that of the badger and this is reflected in European otter scent marking strategy. Kruuk (1992) suggested that sprainting may actually function as a signal to other European otters that a particular stretch of coastline or river, is being, or has already been, utilised. In this way they may act as resource markers to warn off conspecific foragers. Deposits of material with a long lasting odour would be ideal in order to communicate this message to other European otter.

From Table 2 in the results section, the following observations can be made:

1. Seventeen of the 'identified' fatty acids were common to all 3 groups. They were: lauric; myristic; pentadecanoic; palmitic; palmitoleic; stearic; oleic; elaidic; linoleic; linolenic; arachidic; cis-11-eicosenoic; arachidonic; behenic; erucic; tricosanoic and lignoceric.

Spraints consist of faecal material plus anal sac secretion. It might be therefore that those fatty acids found in spraints may come from the anal sac secretion and these 17 shared fatty acids (see Table 2) may be representative of this. Equally, however, these fatty acids may come from the diet of the otter or they may be a combination of fatty acids from the anal sac secretion supplemented by the 'same' fatty acids from the otter diet (this is probably the most likely scenario). It is also possible that some or all of these compounds could carry a message of species identity (through analogue coding).

One fatty acid (heptadecanoic) appeared in captive and wild spraint samples, but not in the anal sac secretion samples (digital coding).

Any fatty acids found in otter spraints and not in the anal sac secretion may be assumed to come from the diet of the otter.

2. Seven fatty acids (capric, tridecanoic, myristoleic, cis-10-pentadecenoic, cis-10-heptadecanoic, γ-linolenic, nervonic) were unique to wild otter spraints and did not appear in the other two groups (digital coding).

Again, these can be assumed to be provided by the European otter diet. As wild European otter have a more varied diet it is reasonable that this would be reflected in their fatty acid range. Individual captive European otters were all an identical diet consisting of day old chicks and a consistent variety of fish. The wild European otter's diet is more varied between individuals and mainly consists of fish (see Chapter 5, Table 15 for further details), but can also include birds, insects, frogs, crustaceans and sometimes small mammals. Scent profiles of wild and captive badger anal sac secretion were found to be significantly different and it was suggested that this may be due to differences in diet or living conditions (Davies et al, 1988). Also, differences in scent profiles for the owl monkey (*Aotus nancymaae*) in terms of age classes (and also sex and family) may be due to a difference in food consumption (Macdonald et al, 2007).

3. Five fatty acids (linolelaidic, cis-11,14-eicosadienoic, cis-8,11,14-eicosatrienoic, heneicosanoic and cis-13,16-docosadienoic) appeared in captive spraint samples and wild anal sac secretion, but not in wild spraint samples (digital coding).

These may be fatty acids that come from the captive European otter's diet and coincidentally appear in the anal sac secretion. Or it may be that these fatty acids 'do' appear in the wild spraint samples, but are at very low levels and consequently are not picked up at the extraction stage or by the analysis. As previously stated wild European otter spraints, as a whole, have less material in than their captive counterparts.

Scent mark weight and percentage fatty acid content

Captive spraint sample weight was significantly higher than that of the wild spraint samples (Captive spraint weight mean = 11.65 g / Wild spraint weight mean = 1.23 g) (See Figure 4). Significant differences were also seen in the (dry weight) % fatty acid content between different forms of European otter scent mark. The lowest % fatty acid content was seen in the wild spraint samples (wild spraint % fatty acid mean = 1.88). The next highest were the captive spraint samples (captive spraint % fatty acid mean = 7.04). The highest % fatty acid content was seen in the wild anal sac secretion samples (wild anal sac secretion % fatty acid mean = 66.2). See Figure 5.

Regular scent marking is important in maintaining a territory and this brings an energetic cost to the territory holder. In theory, selection should favour evolution of costly signalling systems providing a reliable 'cheat-proof' representation about the quality of a signaller (Zahavi, 1975; Grafen, 1990). Laboratory mice, *Mus musculus* have been used to demonstrate that small males are able to maintain dominance over larger competitors by increasing investment in scent marking. This has the associated cost of reduced growth rate and body size (Gosling et al, 2000). Similarly, olfactory signalling in the bank vole, *Clethrionomys glareolus* has been associated with energetic costs (Radwan et al, 2006).

In addition, although faeces may be the ideal scent marking substance due to the minimal energetic cost to the signaller, marking in this way is also constrained by the animal's ability to produce faeces. Studies on the African antelope found that territorial males marked with faeces more often, and with a smaller volume per defecation, than did juvenile males and females. Overall, territorial males regulated their faecal marking behaviour in response to a limited supply of faeces (Brashares & Arcese, 1999). Similarly, wild European otter males produce the smallest spraints (often no more than a few droplets). Females produce bigger spraints, with cubs producing the largest of all (Kruuk, 2006).

It makes energetic sense for a territorial holder to make each mark it deposits as small as possible (provided this is still at a level where the scent mark will be effective).

This study has shown the percentage fatty acid content of both wild and captive *L. lutra* spraints was much lower than that of the wild anal sac secretion alone. This might be explained in terms of energetic cost. The wild anal sac secretion has high % fatty acid content, but only a very small amount is deposited along with faecal material in a spraint (although presumably in amounts large enough to be effective for communication). This makes the most efficient use of the material available. Wild spraint samples tend to be smaller both in terms of percentage fatty acid content and weight than those from their captive counterparts. This might be explained by the availability of food sources. Energy conservation would be more important for wild otters having to forage for their own food, as opposed to captive European otters that are provided with food regularly and reliably. Depositing secretion and faecal material in very small amounts might not be as important for a captive European otter. In addition, captive European otters by definition are confined to a relatively small area compared to the range of a wild European otter. They are not able and have no need to use up huge amounts of energy – they do not need to forage for food or patrol large areas of territory in order to scent mark.

Comparison of identified fatty acids present in captive otter spraint / wild otter spraint / wild otter anal sac secretion

Overlap in fatty acid composition within and between the sex groups in captive European otter spraints

In 'identified' fatty acid overlap terms, female spraints were more similar to other female spraints than they were to male spraints, but male spraints were more similar to female spraints than they were to other male spraints (see Figure 6).

A significant difference was found between the mean overlap *within* the male group and the mean overlap *between* males and females. A significant difference was also found between the mean overlap within the female group and the mean overlap between females and males. However, the overlap within the male group was lower than the overlap between males and females, but the overlap within the female group was higher than the overlap between females and males. Within the 'identified' fatty acids, males and females had an almost identical profile with no real 'sex marker' compounds being

present and so this result is not entirely unexpected (analogue coding). It remains unclear from this part of the results what role, if any, the overall fatty acid profile plays in the identification of sex.

Of the 30 fatty acid identified in this study from the European otter scent marks (*captive spraint / wild spraint / wild anal sac secretion*), 17 were almost universally present (see Table 2) and it is therefore possible that some or all of these compounds could carry a message of species identity.

Differences in absolute and relative content of common compounds between the sexes

1. On a quantitative level there initially appeared to be a difference between the two sexes in that females had a higher absolute content of the five dominant fatty acids found in captive European otter spraints (palmitic, stearic, oleic, linoleic and cis-13,16-docosadienoic) than males (Figure 8). However, none of these differences were found to be statistically significant, although the results for oleic and linoleic acid were closer to being significant than the others.
2. Differences were also appeared to be present in the relative content of these five fatty acids. Females had a higher relative content of oleic, linoleic and cis-13,16-docosadienoic, but males had a higher relative content of palmitic and stearic (Figure 9). Again, these differences were not found to be statistically significant.

If otters were able to detect these possible differences, then a combination of 1 and 2 (above) or alternatively either of these two factors alone could provide a mechanism (based on analogue coding) for distinguishing between the sexes.

Overlap in fatty acid profile within and between individuals

Do the captive European otter spraints carry a message of identity for the individual otter? For 'identified' fatty acids only 50% of the 6 individual European otters studied had a higher overlap within their own samples than their total composition overlapped those of the other individuals (Figure 9). This, plus the fact that no statistical tests were carried out (due to the low number of samples involved) means that no conclusions can

be drawn regarding an individual message. It is important to remember that this specific extraction method does not provide a complete chemical profile of otter scent marks. Other methods will are likely to reveal the presence of a number of other types of compound. Whether the biologically active compounds are present in the fatty acid component of a scent mark is unknown at the present time.

Difference in compound overlap within and between European otter spraint and anal sac secretion groups

Identified fatty acids

This data included just those fatty acids that were positively identified and showed higher overlaps within European otter scent mark groups than between the different groups (Table 5). There was one exception however - the overlap between wild secretion and captive spraints was higher than might be expected - 95.22%. All differences were shown to be statistically significant (see Table 6). This result, in general, illustrates clear differences between the scent marking groups. As previously discussed, dietary differences explain the differentiation.

All fatty acids

This data included the total compound profile highlighted by the extraction process. This showed, in general, a greater distinction between the high overlap level within European otter scent marks and the relatively lower overlap level between the different groups (Table 7). The one exception from the 'identified' fatty acid data (the overlap between wild secretion and captive spraints) now showed a much lower level of overlap - 51.01%. All differences were shown to be statistically significant (see Table 8). This highlights the possible pitfalls of looking at just part of the chemical profile of a scent mark. Other, unidentified fatty acids and indeed other types of compounds altogether may be needed to provide the whole scent picture. Mammal chemical communication is complex and we (unlike European otters whose olfactory apparatus is perfectly suited to deciphering the spraint odour code) are limited by the sophistication of our analytical techniques and apparatus.

Comparisons of spraints from captive European otter individuals using Multi – Dimensional Scaling (MDS)

Identified fatty acids

All initial analyses (absolute and presence or absence) showed no real grouping of individuals or of sexes. Transformation ($\log_{10}$) of the absolute - data showed little improvement in the results. Therefore, no further analyses were carried out. Instead, individual or sex related patterns in the fatty acid profile of captive otter spraints were sought using data for all the fatty acids.

All fatty acids

MDS analysis for absolute content data demonstrated a 'possible' split based on sex. (see Figure 10). The presence or absence data also revealed a possible difference between males and females with the female fatty acid profile clustering within a broader range of male profiles (see Figure 11). However, no significant differences between the sexes were seen for the absolute data, whilst only a weak significant difference was found for the presence or absence data using the ANOSIM analysis.

Looking at all the similarity/dissimilarity tables for absolute content and presence or absence data (Tables 9 and 10) and comparing them to the top 5 dominant acids in terms of absolute content, it is immediately obvious that despite the prominence of these acids in absolute terms, none of them appear in the top 5 acids responsible for similarity within sex groups or dissimilarity between sex groups for the absolute content or presence or absence data. This indicates that although compounds may feature prominently in terms of absolute content or their presence or absence within an otter scent mark, they may not necessarily be the most important for communication.

The presence or absence data had a single dominant acid (with the largest contribution to similarity within the sex groups) in both males and females (arachidic). For the absolute data the dominant acid differed between males and females (males – palmitoleic; females – fatty acid 41).

The largest contributors to dissimilarity between the sexes for the absolute data and the presence or absence data were fatty acid 4 and fatty acid 45.

However, there were some anomalies, as some male samples appeared in the female groupings and vice versa and there are also some outliers from both groups. For example, fatty acid 45 came out as the most dominant compound contributing to dissimilarity between the sex groups in terms of presence or absence. This acid was present within all female samples; however it was also present within 2 of the male samples. More samples are obviously required in order to confirm the distinct male and female patterns suggested here. One explanation may be the possibility that there may have been sampling errors. Most enclosures at sanctuaries have male and female otters housed together. Male and female otters exhibit sexual dimorphism; males are larger than females; have a broader head, plus the differences in their genetalia. When both otters are in view, these differences are obvious, however, when the otters appear one by one then identification can sometimes become problematic. Close observation is necessary in order to avoid mistakes.

So then, despite no statistically significant differences being shown between males and females for both data types, there are some possible differences in the chemicals (e.g., fatty acid 45) which could be revealed through an increase in the number of samples used.

The type of clustering seen with the presence or absence data (i.e., female profiles clustering within a broader male range) was also seen in another study of European otter anal sac secretion (Bradshaw et al, 2001). The main 13 compounds identified from anal sac secretion using 'Coupled Purge and Trap Gas Chromatography - Mass Spectrometry' were used in the analysis (n=25, male=19; female=6). The absolute data were $\log_{10}$ transformed and Principle Component Analysis was used to investigate a male/female pattern. As with this study, the female odour profiles clustered together within a broader male range. Bradshaw et al (2001) suggest that a possible explanation for the absence of a characteristic female odour would be as an extension to the secretive behaviour exhibited

by females around the birth of their young in order to avoid infanticide by male adult otters.

Female European otters can show aggression towards unknown males if they venture too close to young cubs and this is thought to be part of a strategy of protection (Kruuk, 2006). In addition, the remains of small European otter cubs have been found in the stomach of a European otter male (Simpson & Coxon, 2000). Avoidance of infanticidal males is likely to be the main biological advantage of the secretive behaviour shown by European otter females with cubs (Kruuk, 2006). Natal holts are notoriously difficult to locate, they are different from 'normal' holts in that they appear to be unlikely places for a European otter to be found (Kruuk, 2006). Following a foraging bout, a female with cubs will take extreme care not to be seen slipping back into the natal holt, hardly showing herself.

Bradshaw et al (2001) suggest that 'due to the length of cub dependence the female European otter would repel male attention using anal gland odour'. The results from this study would tend to agree with this suggestion.

Sprainting behaviour has been linked with home range advertisement and territorial marking (Erlinge, 1967b, 1968; Kruuk & Hewson, 1978; Macdonald 1980). Territory holders would be very unlikely to kill their own offspring (Ebensperger, 1998) and therefore there would seem to be several strategies involved in order to keep infanticide by unknown males to a minimum: (1) a successful territorial system; (2) aggressive behaviour shown by females towards unknown males (Kruuk, 2006); (3) the secretive behaviour adopted by females with cubs (Kruuk, 2006) supplemented by an absence of a characteristic female odour (Bradshaw et al, 2001).

In addition, during the first months of a cubs life, the female will spraint almost exclusively in water and not on land (this is true of freshwater and coastal European otters) [Kruuk, 2006].

As with the results in the previous sections, no conclusions can be drawn regarding an individual message, but there is a suggestion of possible differences between the sexes, although there was no statistical significance demonstrated. The addition of further data from more individuals might help to resolve these questions.

Comparison of European otter spraints (wild and captive) and anal sac secretion (wild) using Multi- Dimensional Scaling (MDS).

The following sections attempt to decipher the fatty acid codes (digital / analogue / both) for the different scent mark types (captive spraints; wild spraints; wild anal sac secretion).

Identified fatty acids

All initial analyses (absolute and presence or absence) showed some level of grouping of the different scent mark types. The absolute data and the presence and absence data showed three broad groupings divided by scent mark type (wild spraints; captive spraints and wild anal sac secretion – see Figures 12 and 13). A significant overall difference was seen between all scent mark types, together with significant differences between each individual pair of scent mark sample groups (including the wild vs captive spraint sample groups).

In terms of similarity within scent mark groups, palmitic acid appeared to play a major role (see Table 14). For the absolute content data it was the dominant acid responsible for similarity for every scent mark type (captive spraint; wild spraint; wild anal sac secretion). For presence or absence data, the dominant fatty acid for both captive and wild spraints was arachidic acid, but linolelaidic dominated for the wild anal sac secretion scent mark group.

Dissimilarity between groups was not dominated by a specific fatty acid (as seen with the similarity within groups): instead, there were a variety of acids responsible (see Table 15).

These results were largely confirmed by the overlap data for the 'identified' fatty acids (discussed) earlier. Again, dietary differences seem to be the defining factor.

All fatty acids

All MDA analyses for all data types indicated that the composition of the 3 scent mark groups was different. There was a definite separation and close clustering of the 3 scent mark types into groups. A significant overall difference was seen between all scent mark

types, together with significant differences between each individual pair of scent mark sample groups (including the wild vs captive spraint sample groups).

In general, adding further data improved the separation of the different scent mark types, especially when looking at the data on a presence or absence basis.

In terms of similarity within scent mark groups, again, palmitic acid appeared to play a major role (see Table 14). For the absolute content data it was the dominant acid responsible for similarity for every scent mark type (captive spraint; wild spraint; wild anal sac secretion). For presence or absence data, the dominant fatty acid varied from scent mark group to scent mark group (captive spraints: fatty acid 52; wild spraints: lignoceric; wild anal sac secretion; fatty acid 46).

Dissimilarity between groups was not dominated by a specific fatty acid (as seen with the similarity within groups): Instead, there were a variety of acids responsible (see Table 15). For the absolute data the dominant fatty acids in terms of dissimilarity were fatty acid 27 and fatty acid 42. For the presence or absence data the dominant fatty acids in terms of dissimilarity were fatty acid 52 and fatty acid 46. Fatty acid 52 only occured in captive spraints and fatty acid 46 only occurred in wild anal sac secretion (digital coding).

As already discussed in Chapter 4, European otter dietary intake may partly explain the differences in scent mark groups seen in this study. The captive European otter are housed at the Chestnut Centre, a privately owned otter sanctuary with a policy that provides them with a consistent daily diet (see Materials and Methods, Table 1).

The wild European otter diet is subject to seasonal and locational changes and this may have a fundamental influence over the odour profile of European otter spraints. Due to the potentially varied nature of the wild diet, any odour it may lend to a scent mark would be unlikely to contribute to a consistent individual identity, but this may help to identify animals foraging in a particular locality or to discriminate males and females.

Whatever messages are conveyed by the fatty acid profile of *L. lutra* scent mark (possible male / female differences; differences in feeding behaviour) it is unclear whether those seen in ASS were the only important components or whether those fatty acids originating

in the diet serve to supplement these; maybe not as a marker for sex or individuality, but possibly as an indication of fitness and/or residency. A fit territory holder would presumably be eating well and this might be evident to an intruding otter within the scent mark.

As well as intra-specific differences in the fatty acids present in otter scent marks, there may also be inter-specific differences. This possibility was investigated in the next chapter.

Sulphur compounds

Many mustelid anal sac secretions have been shown to contain sulphur compounds. These include those of the stoat, *Mustela erminea* (Crump 1980a); the ferret (Crump 1980b); the mink, *Mustela vison* (Sokolov et al, 1980); the steppe polecat, *Mustela eversmanni* (Zhang et al, 2002) and the weasel, *Mustela nivalis* (Buglass et al, 1991). However, sulphur compounds were not found in the anal sac secretion of the European badger (Davies et al, 1988). Many sulphur compounds are well known microbial products (Sokolov et al, 1980).

This study has revealed the presence of 2 possible sulphur containing compounds in *L. lutra spraint* material: dimethyl sulphide and dimethyl sulphone. Their presence in the analysis of both captive and wild spraints and absence from the analysis of wild anal sac secretion would seem to indicate that these compounds have their origins in the diet.

As previously discussed in Chapter 4, dimethyl sulphide acts as a foraging cue in Antarctic Procellariiform seabirds, (Nevitt et al, 1995). In addition, a similar compound, dimethyl disulphide has been identified in the anal sac secretion of the mink (Sokolov et al, 1980), and the European otter (Bradshaw et al, 2001) and is associated with sexual attraction in the hamster (O'Connell et al, 1979; Petrulis & Johnston, 1995).

CONCLUSIONS

- Low volatility fatty acids (C_{10}-$C_{24.1}$) were identified in all 3 scent mark types - similar to badger and panda, but most other animals have high volatility fatty acids. Possibly a long term signal.
- % fatty acid content of wild and captive European otter spraints was much lower than that of the wild anal sac secretion alone. Wild anal sac secretion has high % fatty acid content, but only a very small amount is deposited along with faecal material in a spraint (although presumably in amounts large enough to be effective for communication). This makes the most efficient use of the material available to the otter. Wild spraint samples were smaller both in terms of percentage fatty acid content and weight than those from their captive counterparts. Energy conservation would be more important for wild otters having to forage for their own food, as opposed to captive otters that are provided with food regularly and reliably. Depositing secretion and faecal material in very small amounts might not be as important for a captive otter.
- Do the captive European otter spraints carry a message of identity for the individual otter? The low number of samples made it impossible to answer this question.
- There was a suggestion of differentiation in spraint fatty acid profiles based on sexual identity. For example, differences were detected in the absolute content of the five dominant 'identified' fatty acids identified in European otter spraints (analogue coding). None of these differences were found to be statistically significant. However, if European otter were able to detect these possible differences then this could provide a mechanism for distinguishing between the sexes.
- Differentiation was seen in European otter scent marking material (captive spraint; wild spraint; wild anal sac secretion). Again, this was demonstrated to be due to a combination of digital and analogue coding, e.g. fatty acid 52 and fatty

acid 46 contributed to digital coding in the dissimilarity between the scent mark groups and palmitic acid contributed to analogue coding.

- As in Chapter 4, part of the explanation for these differences was thought to be found within the otter diet.
- Sulphur compounds are known to have attractant properties in other species. This study has revealed the presence of 2 possible sulphur containing compounds derived from the European otter diet.

FUTURE WORK

- Further identification of the unknown fatty acids seen in this study.
- An assessment of the ability of otters to distinguish between different combinations of fatty acids (i.e., in effect, artificially produced mixtures produced to mimic the fatty acid content of otter spraints). This could either be done through training animals using a system of rewards or using an indirect measure, e.g., an overmarking response.
- An assessment of the possible attractant properties of the sulphur-containing compounds found in this study.

CHAPTER 7

INTER-SPECIFIC DIFFERENCES IN THE FATTY ACID PROFILE OF SPRAINTS FROM FOUR SPECIES OF OTTER: THE EUROPEAN OTTER (*Lutra lutra*); THE CANADIAN RIVER OTTER (*Lontra canadensis*); THE ASIAN SHORT CLAW OTTER (*Aonyx cinerea*) AND THE GIANT OTTER (*Pteronura brasiliensis*)

INTRODUCTION

In this study, the fatty acid constituents of spraint from 4 different otter species (European, *Lutra lutra*; Canadian river, *Lontra canadensis*; Asian short claw, *Aonyx cinerea*; and Giant, *Pteronura brasiliensis*) have been examined, initially by identifying those present. Otters are found in all continents (except Australia and Antarctica) and any overlap in the range of different species is normally only observed along with major differences in diet or habitat (Kruuk et al, 1994). See Chapter 5 for ecological and behavioural information regarding the European otter; the Canadian river otter; and the Asian short-claw otter to supplement the following information on the giant otter (taken from Kruuk, 2006).

Giant otters are found in several South American countries. Principally they are found in the Brazilian Amazon (but are extinct from Argentina) and live in areas with dense forests and slow moving rivers. It is the longest of the otter species measuring up to 2m and weighing in at up to 32 kg. The giant otter has a gregarious nature and has been observed in groups of up to 20 individuals. They generally live, forage and defend territories in these groups and use a variety of communication systems. Unlike most other otter species, the giant otter uses loud calls (even underwater) and postures, however in common with the other species scent communication is also hugely important. Groups of giant otters take up temporary residence alongside the water's edge and spend a large proportion of their time depositing spraints, anal sac secretions, and urine for the benefit of passing otters. All vegetation is removed and the otters scrape, trample, and roll through the resultant mud. Giant otters are almost exclusively piscivorous. They are diurnal and like their European otter cousins that live along freshwater, this activity pattern appears to be governed by the activity patterns of the fish they predate (ie, active at night, but still during the day).

Of the otter species examined in this study, there are 2 that occur sympatrically in Asia: the European (or Eurasian) otter and the Asian short-claw otter (Kruuk et al, 1994). There is no habitat overlap seen with any of the other otter species (the Canadian river otter occurs in North America and the giant otter in Latin America).

Once again, this study used freshly deposited spraints, which include faecal material in addition to anal sac secretion, in order to try and obtain the whole picture of the fatty acid contribution to deposited spraint odour.

MATERIALS AND METHODS

Study animals and sample collection

A total of 19 spraint samples were collected from:

- 6 adult European otters (3 ♂ and 3 ♀). Two spraint samples were collected from each individual immediately following deposition (NB one female provided 3 samples)
- 2 adult Canadian river otters (1 ♂ and 1 ♀). One spraint sample was collected from each individual
- 2 adult Asian short-claw otters (1 ♂ and 1 ♀). One spraint sample was collected from each individual
- 1 adult giant otter (♀). Two spraint samples were collected

All otters were housed at 'The Chestnut Centre', Chapel-en-le-Frith, Derbyshire, UK (a privately owned otter and owl sanctuary).

Samples were placed in airtight, labelled glass containers. These were transported back to the lab the same day over dry ice in an insulated container. Once at the lab the samples were stored at -70°C immediately in order to avoid deterioration prior to analysis. All spraint samples were defrosted in order to take a sub-sample for freeze-drying. Approximately 1.5g of material was removed from each spraint sample. All sub samples were refrozen to -70°C.

Fatty acid extraction procedure – refer to Chapter 6, Materials and Methods.

GC analysis

All spectra were obtained using a Thermo-Finnigan GCQ plus Ion Trap Mass Spectrometer 70ev E1MS using control acquisition software (Thermo-Finnigan version 2.31, pc based).

Temperature programme

The following conditions were used: source Temp: 220°C, Transfer Line: 280°C, Injector Temp 260°C. Separation was performed on DB5-MS column (J & W 30m, 0.316mm I.D., 0.25µm coating) with helium (1ml min^{-1}) as a carrier gas using the following temperature programme: 42°C, held for 5 minutes, then rising at 5°C min^{-1} to 150°C and finally rising at 15°C min^{-1} to 270°C; splitless injection used (split close time: 0 minutes, split open time: 4 minutes). All fatty acids were identified and confirmed by comparison of spectra and retention times to those of standards (Sigma-Aldrich Company Limited, Gillingham, Dorset, UK).

Total ion count (TIC) was used for each of the peaks on the resultant chromatograms, and these values were normalised to calculate the relative content of each compound in a sample. Additionally, within each group the normalised percentages were averaged to provide a mean percentage contribution.

Statistical data analyses

As in prvious chapters, Multi- Dimensional Scaling (MDS) [Primer 5, version 5.2.0] was employed to clarify any underlying trends in the relatedness of compound composition. This enabled comparison of spraints from different species of captive otter (European otter; Canadian river otter; Asian short claw otter; and Giant otter).

The MDS analysis enabled comparison of:

- The spraints from different species of captive otter, using:
    - log_{10} transformation of absolute data
    - presence or absence data

NB this was carried out for ‘identified fatty acids only’ and also for ‘all fatty acids’

SIMPER analysis was again applied to the similarity matrices generated for each scent mark group. This examined the contribution of each chemical to the average Bray-Curtis dissimilarity between the groups of. Additionally the contribution to similarity within a group was determined.

As described in chapters 4 to 6 the Analysis of similarities (ANOSIM) procedure was also carried out using the PRIMER software. This provided a way to test statistically whether there was a significant difference between two or more groups of sampling units (in this case, different species of otter).

Once again, as in previous chapters, the concept of `overlap' between samples was used in comparing the spraint profiles.

The differences found between samples were tested for significance using independent *t*-tests. Levene's test for equality of variances was initially employed to identify the most appropriate *t*-test (equal variances assumed/equal variances not assumed).

RESULTS

Fatty acids extracted

GC analysis of spraint samples from all species of captive otter allowed positive identification of 27 fatty acids (The European otter had 23; the Asian short-claw otter had 23; the Canadian river otter had 26; and the giant otter had 19). The types and mean percentage contributions of these fatty acids varied between the species, highlighted in Table 1 by focussing on the 5 most dominant fatty acids for each species in terms the mean percentage contribution. The sum of these fatty acids contributed >66% of the total identified in each case (European otter >87%; Asian short-claw otter >74%; Canadian river otter >66%; giant otter >72%). Each species had a specific blend of dominant fatty acids that contributed to the fatty acid profile in varying amounts.

Typical spraint chromatographs from all 4 otter species are shown in Figures 1-4.

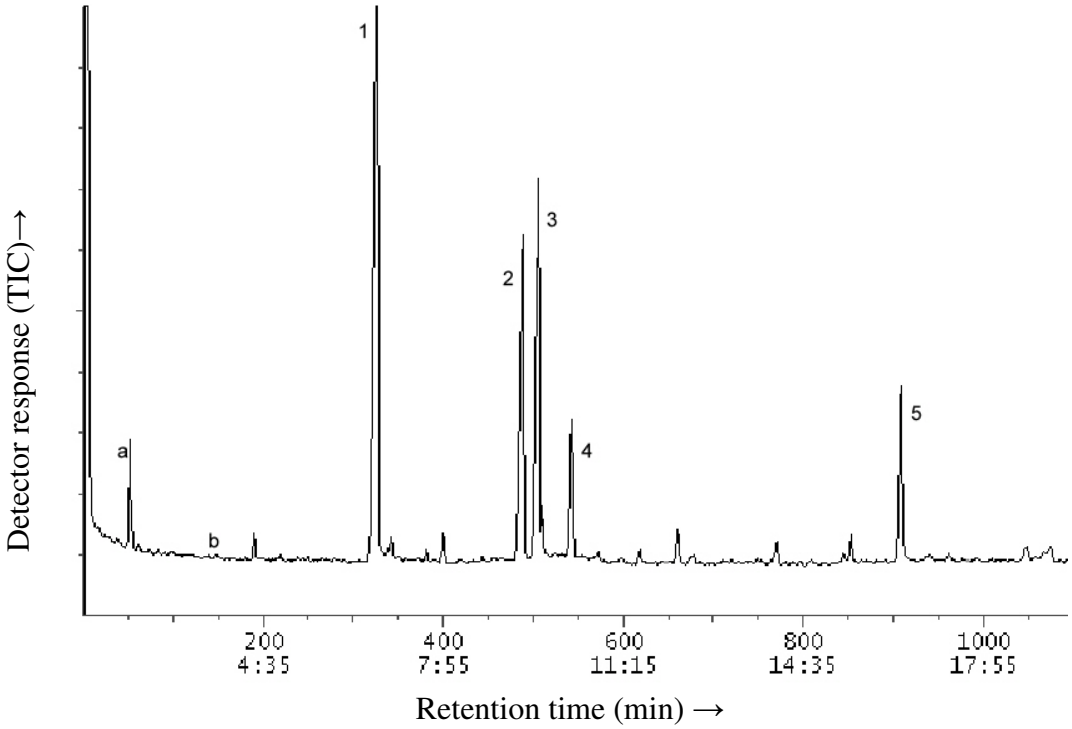


Figure 1. Gas chromatogram of captive **European otter** spraint samples. Numbers indicate the 5 dominant acids (1 = palmitic; 2 = stearic; 3 = oleic; 4 = linoleic; 5 = cis-13,16-docosadienoic). Letters indicate possible sulphur related compounds (a = dimethyl sulphide; b = dimethyl sulphone).

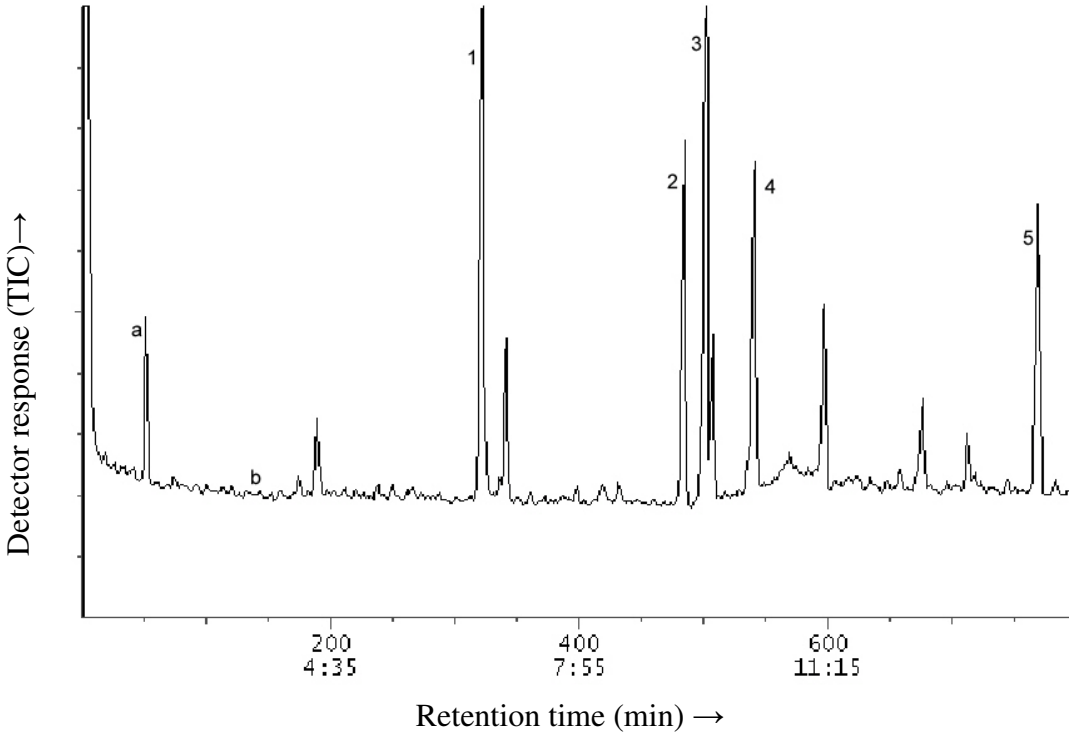


Figure 2. Gas chromatogram of captive **Canadian river otter** spraint samples. Numbers indicate the 5 dominant acids (1 = palmitic; 2 = stearic; 3 = oleic; 4 = linoleic; 5 = arachidonic). Letters indicate possible sulphur related compounds (a = dimethyl sulphide; b = dimethyl sulphone).

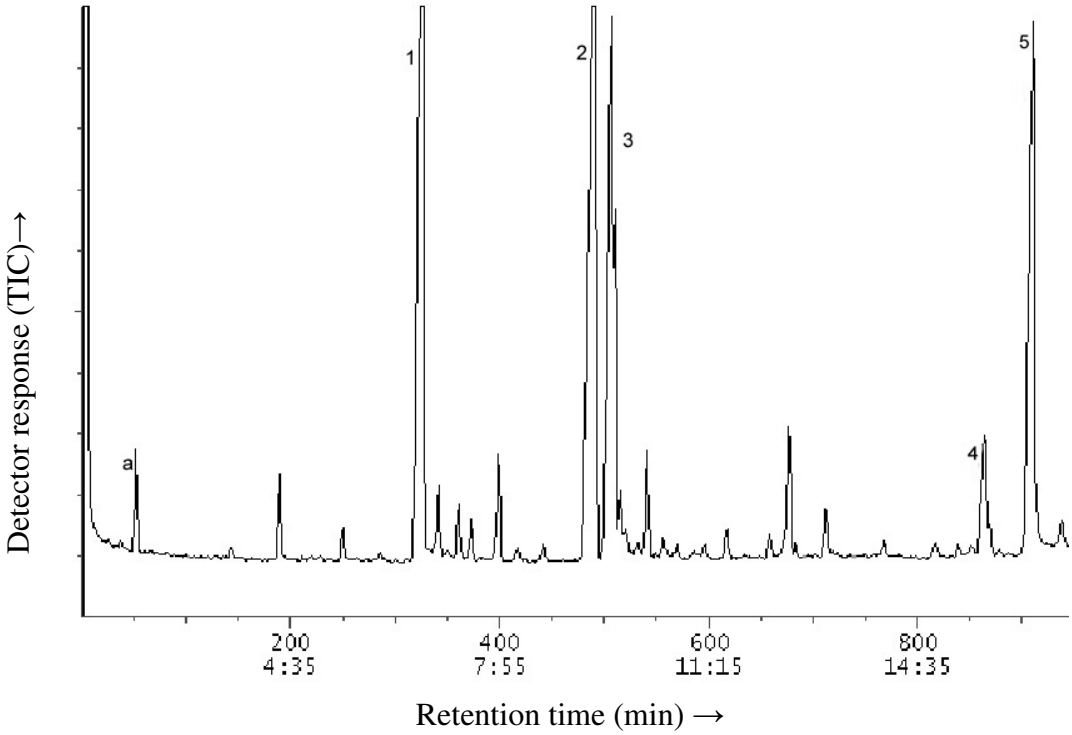


Figure 3. Gas chromatogram of captive **Asian short-claw otter** spraint samples. Numbers indicate the 5 dominant acids (1 =palmitic; 2 =stearic; 3 =oleic; 4 = erucic; 5 = cis-13,16-docosadienoic). Letters indicate possible sulphur related compounds (a = dimethyl sulphide).

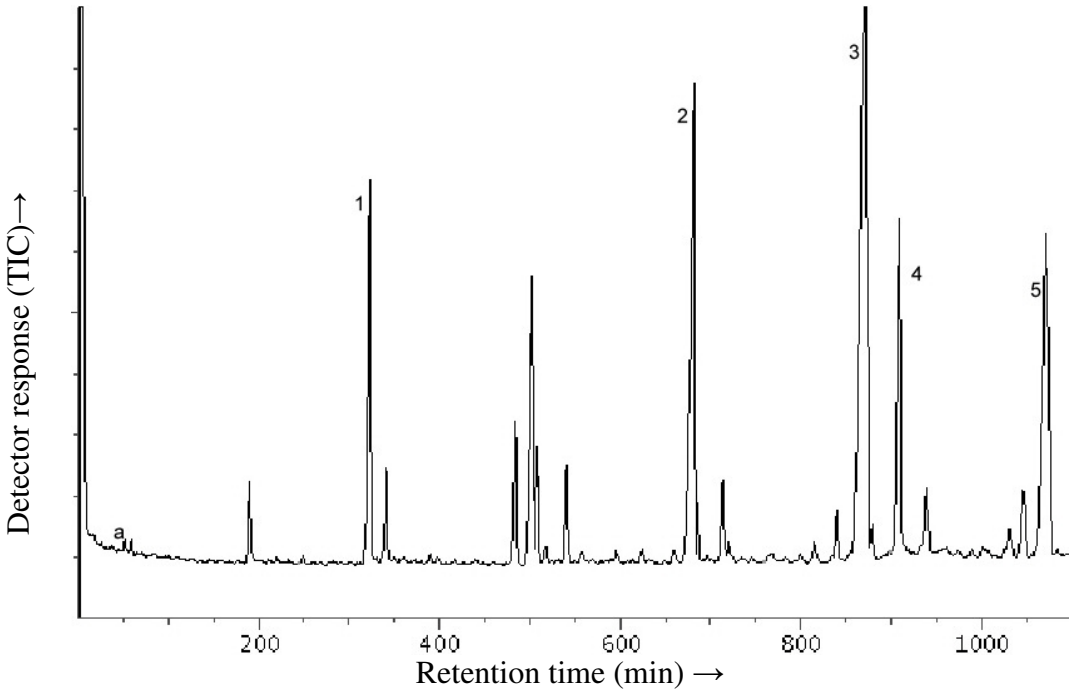


Figure 4. Gas chromatogram of captive **giant otter** spraint samples. Numbers indicate the 5 dominant acids (1 = palmitic; 2 = cis-11-eicosenoic; 3 = erucic; 4 = cis-13,16-docosadienoic; 5 = nervonic). Letters indicate possible sulphur related compounds (a = dimethyl sulphide).

Table 1: Comparison of identified fatty acids present in the spraint material of 4 captive species of otter: *L.lutra; A.cinerea; L.canadensis* and *P.brasiliensis*. NB – units: % of identified fatty acids.

	Fatty acid	*C chain*	*Ret. Time (min)*	**European %**	*St.Dev.*	**Asian %**	*St.Dev.*	**Canadian %**	*St.Dev.*	***giant %***	*St.Dev.*
1	**lauric**	C12	02:56	**0.08**	0.11			**0.22**	0.09		
2	**myristic**	C14	04:26	**0.79**	0.31	**1.76**	0.79	**3.13**	1.49	**1.99**	0.54
3	**myristoleic**	C14.1	04:48			**0.23**	0.21				
4	**pentadecanoic**	C15	05:28	**0.22**	0.16	**0.54**	0.09	**0.55**	0.12	**0.30**	0.16
5	**palmitic**	C16	06:40	**37.19**	6.37	**22.28**	0.12	**26.10**	15.33	**9.12**	1.63
6	**palmitoleic**	C16.1	06:59	**0.95**	0.52	**1.63**	0.85	**3.18**	0.66	**2.38**	0.75
7	**heptadecanoic**	C17	07:58	**1.14**	0.51	**1.22**	0.81	**0.73**	0.57	**0.18**	0.08
8	**cis-10-heptadecanoic**	C17.1	08:19			**0.17**	0.16	**0.66**	0.11		
9	**stearic**	C18	09:19	**17.17**	2.99	**17.25**	10.11	**12.36**	4.46	**4.07**	1.41
10	**oleic**	C18.1	09:39	**17.20**	4.38	**12.87**	3.51	**15.39**	3.76	**8.52**	1.72
11	**elaidic**	C18.1	09:42	**1.73**	0.73	**5.15**	0.71	**1.69**	0.34	**2.6**	1.11
12	**linoleic**	C18.2	10:17	**4.92**	0.84	**5.06**	4.59	**7.47**	4.31	**2.32**	0.44
13	**linolelaidic**	C18.2	10:43	**0.29**	0.13	**0.17**	0.24	**0.34**	0.17		
14	**γ-linolenic**	C18.3	10:59			**0.12**	0.17	**0.09**	0.12		
15	**linolenic**	C18.3	11:12	**0.20**	0.23	**0.02**	0.02	**3.57**	1.15		
16	**arachidic**	C20	12:19	**1.32**	0.77	**0.65**	0.43	**0.92**	0.50	**0.31**	0.08
17	**cis-11-eicosenoic**	C20.1	12:38	**0.41**	0.17	**4.03**	2.16	**3.43**	0.64	**13.44**	1.19
18	**cis-11,14-eicosadienoic**	C20.2	13:16	**0.23**	0.15	**1.30**	0.74	**0.72**	1.02	**0.35**	0.24
19	**cis-8,11,14-eicosatrienoic**	C20.3	13:40	**0.25**	0.28	**0.28**	0.39	**0.28**	0.27		
20	**heneicosanoic**	C21	13:46	**0.10**	0.10			**0.2**	0.07	**0.18**	0.19
21	**arachidonic**	C20.4	14:06	**1.47**	0.54	**2.39**	2.93	**5.61**	4.79	**0.28**	0.07
22	**behenic**	C22	15:28	**1.14**	0.68	**0.36**	0.32	**0.93**	0.59		
23	**erucic**	C22.1	15:41	**0.18**	0.19	**5.30**	2.89	**3.81**	2.01	**25.77**	7.69
24	**cis-13,16-docosadienoic**	C22.2	16:28	**11.38**	12.98	**16.49**	0.47	**6.09**	7.95	**10.01**	1.33
25	**tricosanoic**	C23	17:00	**0.67**	0.68	**0.72**	0.11	**1.48**	1.42	**1.99**	0.37
26	**lignoceric**	C24	18:43	**0.95**	0.52			**1.05**	0.02	**2.18**	0.23
27	**nervonic**	C24.1	19:17					**0.00**	0.00	**14.02**	1.31

Highlights indicate the 5 most dominant acids for each species in terms of the mean percentage contribution

Overall, 16 fatty acids were common to all species (numbers: 2, 4, 5, 6, 7, 9, 10, 11, 12, 16, 17, 18, 21, 23, 24, 25) and only one fatty acid (myristoleic acid in the Asian short-claw otter) was found to be 'species-specific' (see Table 1).

Two possible sulphur based compounds were also identified using the MS library (these were not confirmed with standards). Dimethyl sulphide (eluted at 02:08) was common to all species and dimethyl sulphone (eluted at 03:43) was only found in the European otter and Canadian river otter samples.

The spraints from each species also had a number of unidentified compounds (see Table 2).

Table 2. Unidentified spraint compounds

species	unidentified compounds	possible fatty acids	unknown
European	15	14	1
Canadian river	28	19	9
Asian short-claw	23	22	1
Giant	31	27	4

Scent mark weight and percentage fatty acid content

Mean spraint sample weight for 2 of the 4 species (European and Canadian river) was fairly similar at 11.5 g and 10.82 g respectively. Asian short claw mean spraint sample weight was lower at 7.02 g and Giant otter mean spraint sample weight was higher at 21.26 g (see Figure 5).

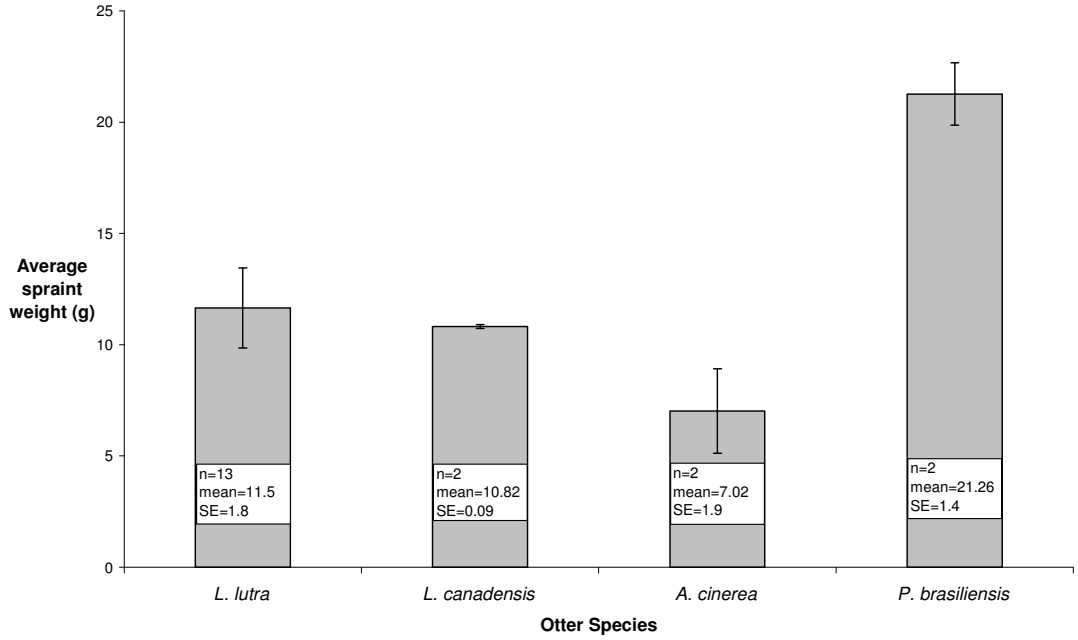


Figure 5. Otter spraints: comparison of weight (g)

The differences in spraint weights between Asian short-claw otters and Canadian river otter and between the giant otter and the Canadian otter were statistically significant (see Table 3 in **bold**). All other differences were statistically insignificant.

Table 3. Comparison of % fatty acid content in different otter species – independent *t*-test results

Comparison	*t value*	*df*	*Sig.(2-tailed)*
European vs Canadian	0.15	13	0.89
European vs Asian	0.96	13	0.36
European vs Giant	-2.09	13	0.057
Canadian vs Asian	2.05	2	0.18
Canadian vs Giant	-7.42	2	**0.018**
Asian vs Giant	-6.13	2	**0.026**

The average (dry weight) percentage fatty acid content of the spraint samples for all 4 species was calculated. The lowest % fatty acid content was seen in the Canadian river samples at 3.61%. The next highest were the Asian short-claw samples at 4.42%. This was followed by the Giant samples at 6.33% and the highest % fatty acid content was seen in the European samples at 7.22% (see Figure 6).

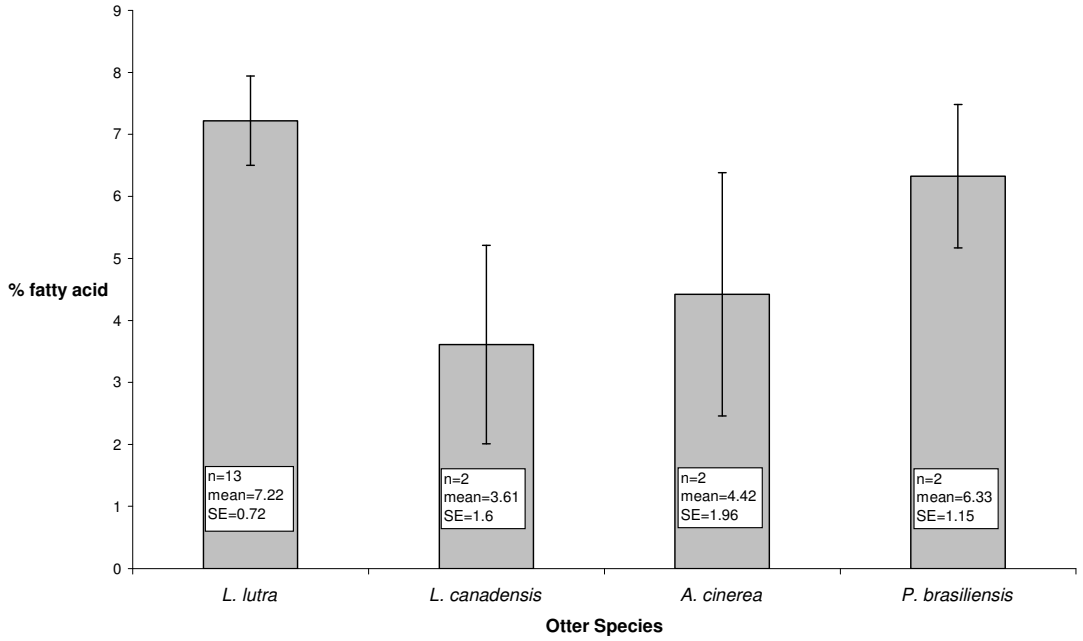


Figure 6. (Dry weight) percentage fatty acid content (with standard error bars) of otter spraint material: comparison of the European, the Canadian river, the Asian short claw and the Giant otter.

All differences were statistically insignificant (see Table 4).

Table 4. Comparison of % fatty acid content in different otter species – independent *t*-test results

Comparison	*t value*	*df*	*Sig.(2-tailed)*
European vs Canadian	1.84	13	0.089
European vs Asian	1.41	13	0.18
European vs Giant	0.46	13	0.65
Canadian vs Asian	-0.32	2	0.78
Canadian vs Giant	-1.38	2	0.30
Asian vs Giant	-0.84	2	0.49

Comparisons of spraints from different otter species using Multi- Dimensional Scaling (MDS)

Identified fatty acids **- absolute content data, log_{10} transformed** 1 way Global ANOSIM [R=0.92, p=0.001]

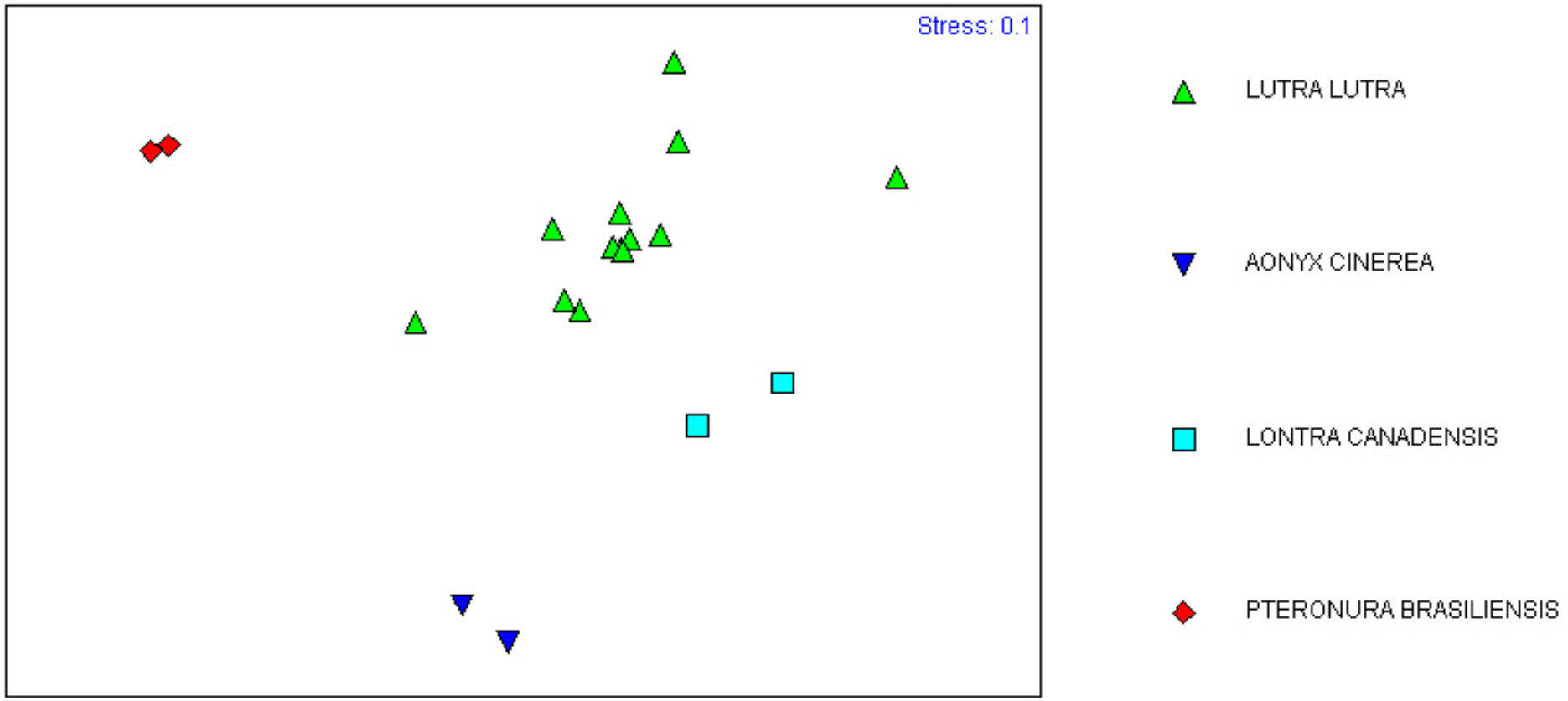


Figure 7. Multi Dimensional Scaling diagram showing relatedness between captive otter species, based on log_{10} transformed absolute fatty acid data. Global ANOSIM R value indicates an overall significant difference between the otter species.

Identified fatty acids - *presence or absence data* 1 way Global ANOSIM [R=0.92, p=0.001]

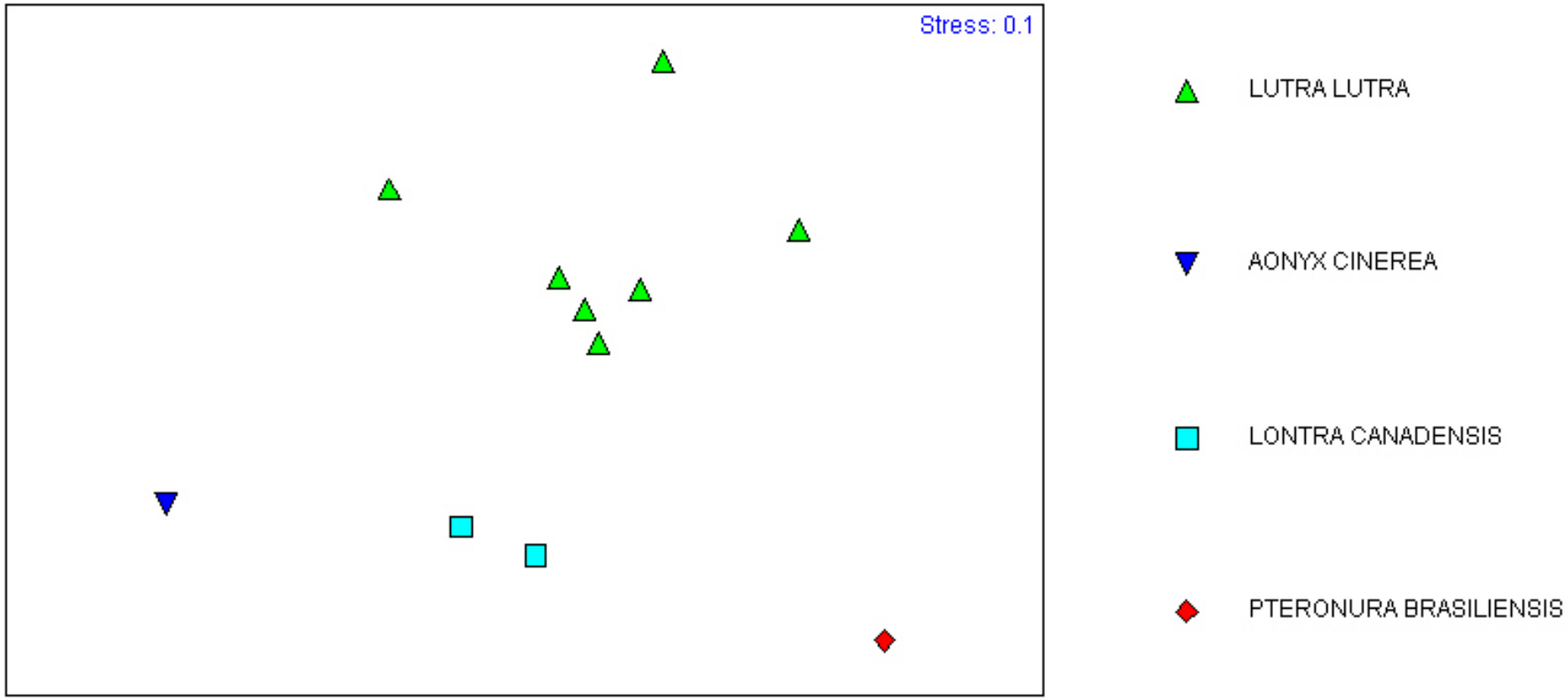


Figure 8. Multi Dimensional Scaling diagram showing relatedness between captive otter species, based on presence or absence fatty acid data. Global ANOSIM R value indicates an overall significant difference between the otter species.

***All fatty acids* - absolute content data, log_{10} transformed** 1 way Global ANOSIM [R=1, p=0.001]

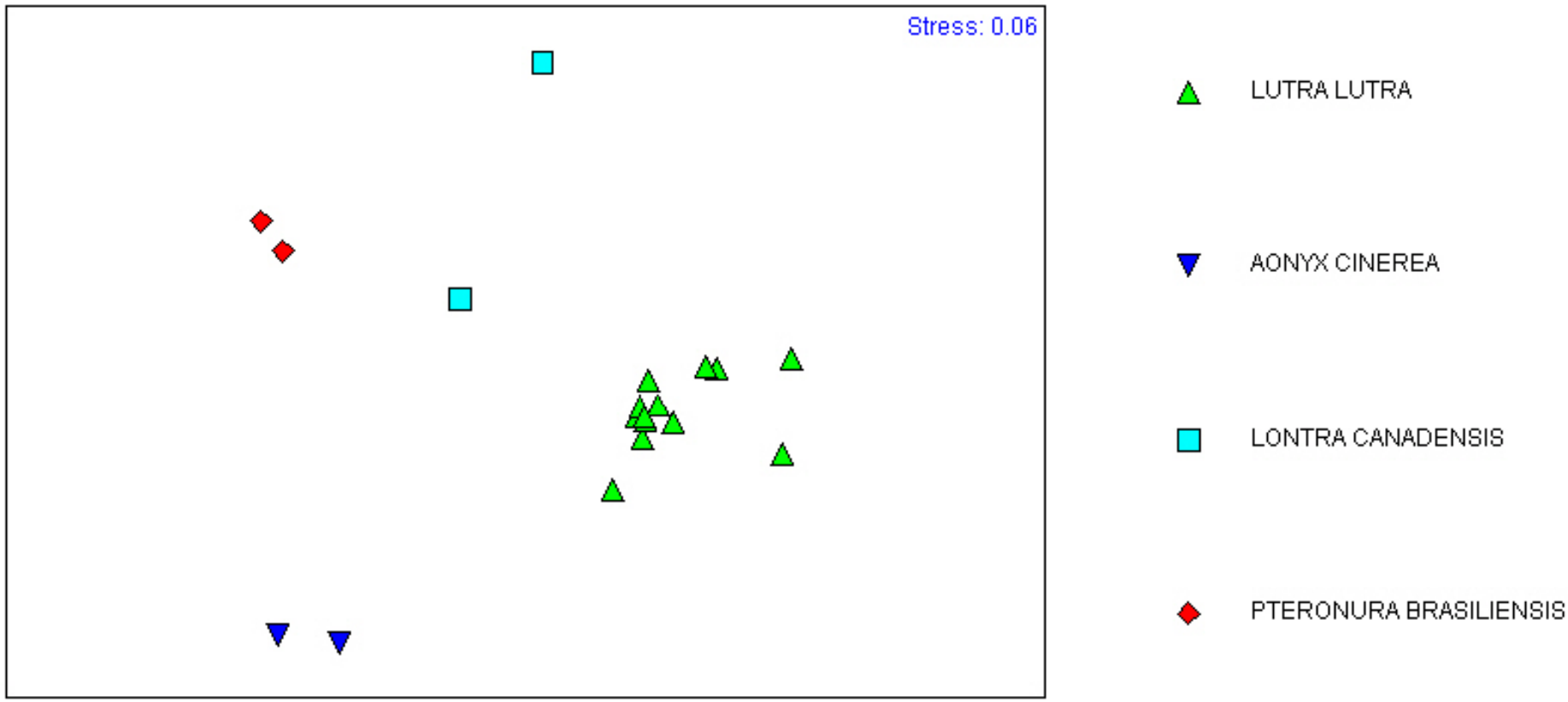


Figure 9. Multi Dimensional Scaling diagram showing relatedness between captive otter species, based on log_{10} transformed absolute fatty acid data. Global ANOSIM R value indicates an overall significant difference between the otter species.

***All fatty acids* - presence or absence data** 1 way Global ANOSIM [R=1, p=0.001]

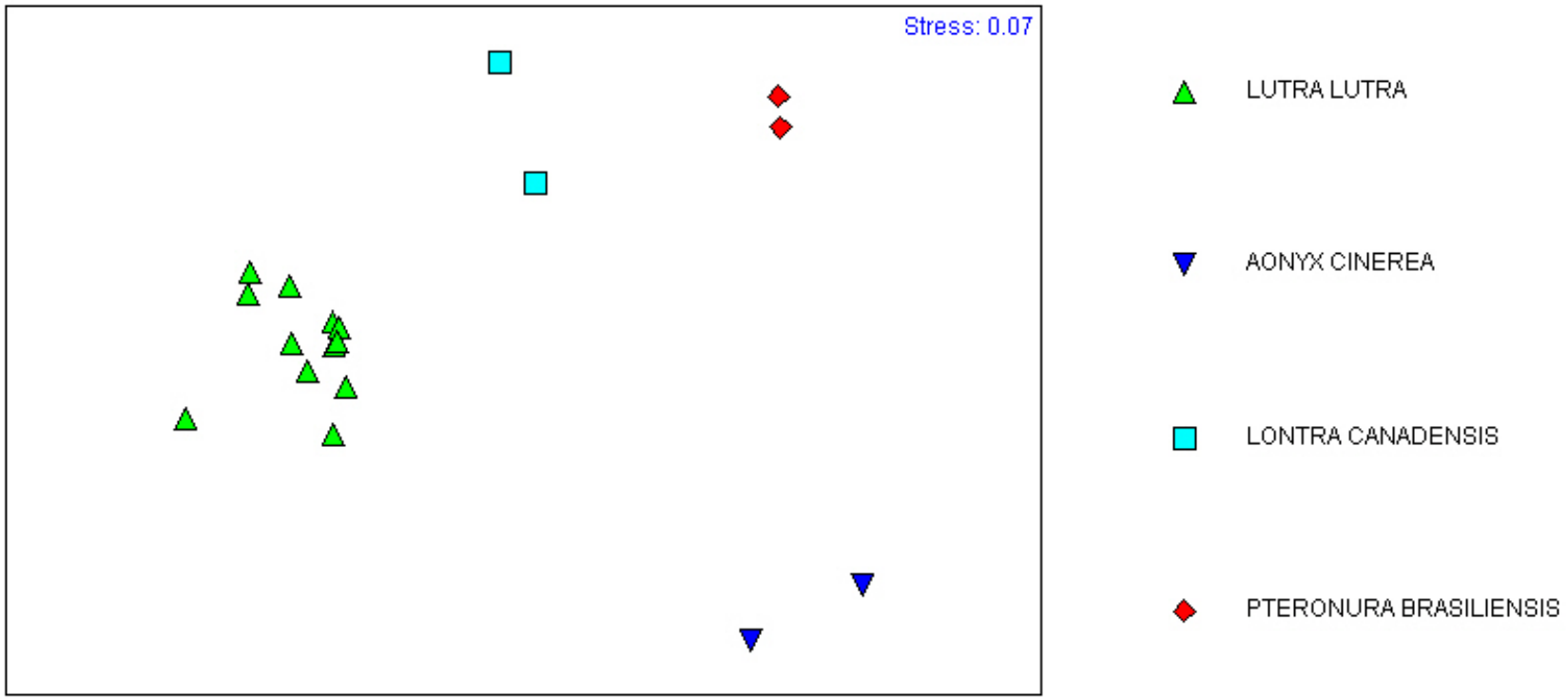


Figure 10. Multi Dimensional Scaling diagram showing relatedness between captive otter species, based on presence or absence fatty acid data. Global ANOSIM R value indicates an overall significant difference between the otter species.

All MDS analyses using both identified and all fatty acid data (absolute, presence or absence) showed a similar result; separation between the 4 captive otter scent mark groups (European otter; Canadian river otter; Asian short-claw otter; Giant otter). However, the analyses using all fatty acid data gave a clearer result than when the identified fatty acids were analysed alone.

These results were confirmed by the Global ANOSIM results for both data types (absolute and presence or absence) and for both the identified and all fatty acids analyses. A significant overall difference between otter species was seen. Pairwise comparisons between separate pairs of otter species were also made. Table 5 shows the results of these comparisons for both data types.

Table 5. Pairwise Anosim comparison of otter scent mark types (*Abs* – Absolute data; *P/A* – Presence or Absence data)

	Groups	R statistic		p value		Possible Permutations	Actual Permutations
		Abs	*P/A*	*Abs*	*P/A*	(for both data types)	(for both data types)
	Identified fatty acids						
1	*European, Asian*	0.996	0.984	0.01	0.01	105	105
2	*European, Canadian*	0.752	0.762	0.01	0.01	105	105
3	*European, Giant*	1	0.965	0.01	0.01	105	105
4	*Asian, Canadian*	1	1	0.33	0.33	3	3
5	*Asian, Giant*	1	1	0.33	0.33	3	3
6	*Canadian, Giant*	1	1	0.33	0.33	3	3
	All fatty acids						
7	*European, Asian*	1	1	0.01	0.01	105	105
8	*European, Canadian*	1	1	0.01	0.01	105	105
9	*European, Giant*	1	1	0.01	0.01	105	105
10	*Asian, Canadian*	1	1	0.33	0.33	3	3
11	*Asian, Giant*	1	1	0.33	0.33	3	3
12	*Canadian, Giant*	1	1	0.33	0.33	3	3

All pairwise comparisons show a difference between each pair of otter species with R values equal to or approaching 1 but not all comparisons were significant (comparisons 4 to 6 and 10 to 12). However, significance is strongly influenced by the number of permutations, particularly when the number of possible permutations is low. Some of the comparisons above had very low numbers of permutations, for

example - comparisons 4, 5, 6, 10, 11, 12. These all had an R value of 1 (indicating a large difference between species), but a p value of 0.33. Here, the significance level may be misleading as there are only 3 permutations possible so significance can never get above p=0.33 regardless how strong the difference. Conversely, if a high number of permutations is seen then you may get a significant result in terms of the p value, but if R is small then this indicates a relatively minor or trivial (albeit significant) difference.

Further analysis was now carried out in order to ascertain which chemicals might be most responsible for the separation seen. Tables 6, 7 and 8 summarise the % similarity within and the dissimilarity between the sex groups for each data type. Tables 9, 10 and 11 show the chemicals most responsible for these similarities and dissimilarities.

SIMPER analysis

Similarity / dissimilarity summary tables

Comparison of spraints different otter species

Table 6 summarises the average percentage similarities within the otter species groups.

Table 6. Summary of average (%) similarity between otter species groups in terms of spraint fatty acid profile, based on log_{10} transformed absolute and presence or absence fatty acid data.

	Data type	Average % Similarity			
		European	*Canadian*	*Asian*	*Giant*
Identified fatty acids	*absolute content* data, log_{10} transformed	93.98	93.44	94.16	98.83
	presence or absence data	96.39	96.00	100.00	100.00
All fatty acids	*absolute content* data, log_{10} transformed	91.50	81.30	91.76	95.99
	presence or absence data	92.69	85.90	87.89	97.52

Tables 7 and 8 summarise the fatty acids that have the largest contribution to *dissimilarity* between the scent mark groups for each data type.

Identified fatty acids

Table 7. Summary of average (%) dissimilarity between otter species groups in terms of 'Identified' spraint fatty acid profile, based on $\log_{10}$ transformed absolute and presence or absence fatty acid data.

	European	*Canadian*	*Asian*
Canadian	10.76 8.20	-	-
Asian	16.30 13.47	14.91 12.55	-
Giant	16.29 12.42	17.49 13.34	27.14 20.93

All fatty acids

Table 8. Summary of average (%) dissimilarity between otter species groups in terms of 'All' spraint fatty acid profile, based on $\log_{10}$ transformed absolute and presence or absence fatty acid data.

	European	*Canadian*	*Asian*
Canadian	25.63 21.14	-	-
Asian	32.08 31.31	32.45 30.38	-
Giant	33.11 30.50	28.17 24.53	35.48 33.91

Tables 7 and 8 show that for most data types the Giant otter scent marks are relatively dissimilar to the other otter species.

Table 9 summarises the dominant chemicals responsible for similaritiy within species scent mark groups.

Table 9. Summary of fatty acids with the largest contribution to similarity within the species groups

	Data type	Average % Similarity *European*	*Canadian*	*Asian*	*Giant*
Identified fatty acids	*absolute content* data, log_{10} transformed	palmitic (6.11)	palmitic (5.13)	palmitic (5.57)	erucic (5.97)
	presence or absence data	linolenic (4.69)	linolenic (4.17)	linolenic (4.35)	arachidic (5.00)
All fatty acids	*absolute content* data, log_{10} transformed	palmitic (4.11)	palmitic (4.08)	palmitic (3.36)	erucic (2.94)
	presence or absence data	fatty acid 28 (3.21)	fatty acid 28 (3.23)	fatty acid 28 (3.11)	fatty acid 27 (2.43)

Identified fatty acids

Table 10 summarises the dominant chemicals responsible for dissimilarity within species scent mark groups

Table 10. Summary of average dissimilarity between otter species groups in terms of 'Identified' spraint fatty acid profile, based on log_{10} transformed absolute and presence or absence fatty acid data.

	European	*Canadian*	*Asian*
Canadian	cis-10-heptadecanoic (18.81) nervonic (6.8)	-	-
Asian	lignoceric (13.49) lignoceric (16.45)	lignoceric (13.67) heneicosanoic (16.61)	-
Giant	nervonic (17.51) linolenic (19.12)	linolenic (13.27) linolenic (16.67)	nervonic (14.24) linolenic (11.11)

All fatty acids

Table 11. Summary of average dissimilarity between otter species groups in terms of 'All spraint fatty acid profile, based on log_{10} transformed absolute and presence or absence fatty acid data.

	European	*Canadian*	*Asian*
Canadian	fatty acid 20 (23.61) fatty acid 38 (6.8)	-	-
Asian	fatty acid 23 (5.25) lignoceric (4.56)	fatty acid 23 (5.06) fatty acid 30 (4.54)	-
Giant	nervonic (4.78) fatty acid 29 (4.33)	fatty acidb37 (5.29) fatty acid 29 (5.22)	fatty acid 23 (4.13) fatty acid 27 (3.75)

DISCUSSION

In this study, the fatty acid content of the spraints of 4 species of captive otter (European; Canadian river; Asian short-claw; and Giant) has been investigated.

For inter-specific communication through scent marking to work effectively then an animal must, at least, be able to recognize the odour of its own species. The scent marks of each otter species could easily be distinguished by the human nose, e.g., the distinctive musky odour of European otter spraints was in stark contrast to the more objectionable odour of Asian short-claw spraints, with the odour of the Canadian river otter possessing elements of both. Similarly, the much stronger smelling odour of the giant otter spraints was instantly recognisable. All this suggests the possibility that one of the basic functions of spraint odour may be to signal species identity.

As discussed in Chapter 5, the signalling of species identity could be achieved either through unique scent components (digital coding) or a 'blend' of common scent components (analogue coding) or even a combination of the two.

Fatty acids extracted

A total 27 fatty acids were identified within the spraints of the four captive otter species groups were examined:

- **European otter** (23 Fatty Acids identified in the range C_{12}-C_{24})
- **Canadian river otter** (26 Fatty Acids identified in the range C_{12}-$C_{24.1}$)
- **Asian short-claw otter** (23 Fatty Acids were identified in the range C_{14}-C_{23})
- **Giant otter** (19 Fatty Acids were identified in the range C_{14}-$C_{24.1}$)

All those identified were high molecular weight long-chain acids (C_{12}-$C_{24.1}$) of low volatility. Although volatile fatty acids were not detected, molecular weights ranged from 200.32 – 366.62 with 15 of the total 27 identified fatty acids (C_{12}-$C_{18.3}$) falling below a molecular weight of 300 – the upper limit for airborne pheromones (Bradbury & Vehrencamp 1998).

Other, unidentified fatty acids were also extracted:

- 14 in the European otter spraints
- 22 in the Canadian river otter spraints
- 19 in the Asian short-claw otter spraints
- 27 in the Giant otter spraints

Studies on mustelid anal sac secretion have revealed the presence of fatty acids in only two other species; the mink (*Mustela vison*) and the European badger (*Meles meles*). In the mink, all fatty acids detected were volatile and none were individually identified (Sokolov et al, 1980). However, in the badger, only long chain, high molecular weight acids were found in a similar range (C_{14}-C_{24}) to that found in the captive European otter spraint material (C_{12}-$C_{24.1}$) (Davies et al, 1988). Ten of the fifteen acids identified in the badger anal sac secretion matched those found in the European otter spraint material; ten matched those found in Canadian river otter spraint material and ten matched those found in Asian short-claw otter spraint material (NB the ten fatty acids differed slightly in each case – see Table 1). Lastly, eight of the fifteen acids identified in badger anal sac secretion matched those found in Giant otter spraint material.

In the badger samples the five most dominant fatty acids in terms of mean percentage contribution contributed >49% of the total concentration of fatty acids. In this study the figures were >87% for the European otter spraint samples; >66% for the Canadian river otter spraint samples; >74% for the Asian short-claw otter spraint samples and >72% for the Giant otter spraint samples (see Table 12).

Table 12. Comparison between dominant fatty acid content of European badger anal sac secretion and spraint material from 4 otter species.

	Badger (Davies et al, 1988) Wild anal gland secretion	Otter spraint material			
		European	*Canadian*	*Asian*	*Giant*
Number of Fatty Acids identified	15	23	26	23	19
Fatty Acid range	C_{14}-C_{24}	C_{12}-C_{24}	C_{12}-$C_{24.1}$	C_{14}-C_{23}	C_{14}-$C_{24.1}$
5 Most dominant Fatty Acids	1. palmitic 2. stearic 3. oleic 4. linoleic 5.eicosatrienoic	1. palmitic 2. stearic 3. oleic 4. linoleic 5. cis-13,16-docosadienoic	1. palmitic 2. stearic 3. oleic 4. linoleic 5. arachidonic	1. palmitic 2. stearic 3. oleic 4. erucic 5. cis-13,16-docosadienoic	1. palmitic 2. cis-11-eicosenoic 3. erucic 4. cis-13,16-docosadienoic 5. nervonic
Dominant Fatty Acid percentage contribution	>49%	>87%	>66%	>74%	>72%

Only 1 fatty acid (palmitic) appeared in the dominant acids of all the mustelid species in Table 1. The badger fatty acids matched 4 of those found in the European otter and the Canadian river otter; 3 in Asian short-claw otter and only 1 in Giant otter. Amongst the otter species, the European and the Canadian river appear to be very similar to each other, matching 4 of the 5 dominant fatty acids. The Asian short-claw otter also matches 4 of the 5 dominant fatty acids with the European otter, and 3 with the Canadian river otter. The Giant otter matches 3 of the 5 dominant fatty acids with the Asian short-claw; 2 with the European and just one with the Canadian river otter. Examining the 'identified' fatty acids in isolation, only one species possesses a unique fatty acid (myristoleic acid in Asian short-claw — see Table 1).

As with the compounds found (using SPME) in Chapter 5 one explanation for the common peaks could be that they represent an overall 'otter marker' and this might then be supplemented by different blends of chemicals together with unique chemicals making up a 'species marker'. It might be of course that unique chemicals may be enough to identify a species (digital coding), e.g., the presence of myristoleic acid in a spraint might be enough to indicate Asian short-claw otter. The other species of otter in this study, however, have no unique chemicals and so would have to rely on unique blends of chemicals to make up a typical species odour (also digital coding) or an odour based upon different amounts of the same chemicals (analogue coding)..

Davies et al (1988) suggested that badger anal gland secretion, due to the presence of long chain fatty acids of low volatility would be suited to long term signalling and

may function in badger territoriality. This could also be true of the fatty acid content of the otter spraint samples, as a long term intra- and inter-specific signal.

European otters, however, mark their territory in a markedly contrasting manner to that of other carnivorous mammals, e.g., badger and spotted hyaenas (*Crocuta crocuta*), where boundary scent marking is extensive (Kruuk, 1972, 1978b, 1989; Mills, 1990). Instead of marking all the boundaries of their territories, otters limit their scent marks to particular areas within them (Kruuk, 1992).

NB the spraint marking behaviour of the Canadian river otter is similar to that of the European otter, marking along their linear river territories, although the spraint odour very different (Kruuk, 2006). The sprainting behaviour of both Asian short-claw and the Giant otter differs from this model. Asian short-claw otters spraint some distance from the water and in the hollows between boulders. Giant otters take up temporary residence along the water's edge, remove all vegetation and take up a lot of time sprainting and urinating in order to convey their olfactory message over a large area of water. Another possibly important difference between the species concerns solitary or group living. European otters are the most solitary of the 4 species, when more than one is seen together it is usually a mother with her cubs. Whereas, it is usual to observe the Canadian river otter, the Asian short-claw otter and the Giant otter living as groups with mean numbers of individuals standing at 4, 5 and 10 respectively (Kruuk, 2006). The specific olfactory messages conveyed in these spraints may differ from species to species, perhaps in part, due to the differences in the level of contact between individuals. It may also be that the overall message conveyed by the different otter species is essentially the same regardless of the number of group size, i.e., resource marking in order to warn off conspecific foragers. This message may also, of course, function inter-specifically and so it would make sense for chemicals within the scent marks of different territorial species to have matching elements to them. The presence of certain common chemicals could represent a typical 'otter' odour.

Scent mark weight and percentage fatty acid content

Mean spraint sample weight was higher for the Giant otter (21.26 g) when compared to the mean spraint sample weight of the other otter species. The European otter and the Canadian river otter had mean spraint sample weights that were fairly similar: European otter – 11.5 g; Canadian river otter – 10.82 g and Asian short-claw otter had

the smallest mean spraint sample weight of 7.02 g (see Figures 5). However, only the differences in spraint weights between Asian short-claw otter and Canadian river otter and between Giant otter and Canadian river otter were statistically significant.

Mean spraint weight is correlated with the weight of each otter species (see Table 13).

Table 13. Comparison of otter weight to mean spraint weight

Otter Species	*Asian*	*Canadian*	*European*	*Giant*
Otter weight (kg)	4-5†	7-9†	8-10†	32*
Spraint weight (g)	7.02	10.82	11.65	21.27

- - Kruuk, 2006 / † - Chanin, 1993

So, from these results, larger otter species produce larger spraints. However, Kruuk (2006) states that Asian short-claw otter spraints are larger (8 cm long and 3 cm in diameter) than those from other otter species sharing the same environment (Kruuk, 2006). The Asian short-claw otter is the smallest otter in the world (Stone, 1992) and so it might be expected that they would deposit the smallest spraints (as in this study). One reason this might not be the case could involve diet. All 4 species of otter used in this study are captive animals and feed on the same types of food, albeit in differing amounts. The wild Asian short-claw otter spraint may be bigger than those of other sympatric species due to the consumption of a different diet. Asian short-claw otters are known to primarily consume molluscs, crabs and other small aquatic animals (Stone, 1992) and indeed their spraints are highly conspicuous with pieces of white crab carapax (Kruuk, 2006). This is a very different diet to those other otter species sharing the same environment – the European otter feeds primarily on fish, which makes up 70-95% of their diet (Woodroffe, 1994) and the smooth otter, *Lutra perspicillata* diet is also dominated by large fish (Kruuk, 2006). Both these otters are known to occur sympatrically with the Asian short-claw otter (Kruuk et al, 1994).

In Chapter 6 some European otter fatty acids were attributed to the wild diet and it was speculated that they may be important, for example, in the identification of territory holders with exclusive access to superior feeding grounds. It is also possible

that odour from faecal material in scent marks may help to differentiate between sympatric species that have some level of dietary separation.

Another possible reason for the relatively large size of Asian short-claw otter spraints may be to do with spraint function. There are criteria commonly used to discriminate between scent marks and excretory eliminations. These criteria state that when scent marks are used for olfactory communication:

- eliminations are small in volume ('token' amounts)
- they are deposited at specific and traditionally used sites that are conspicuous and easily discovered
- their odour is a great source of interest

Asian short claw otter spraints are relatively large and they are often deposited away from the water and in the hollows between boulders rather than at a high point (Kruuk, 2006). Both the European otter and the smooth otter deposit their spraints along the water's edge and in a conspicuous manner (Kruuk, 2006). It has been hypothesized that the European otter may not be territorial in the classic sense with sprainting behaviour seeming to simply advertise an individual's presence in order to provide personal foraging space (Durbin, 1989; Kruuk, 1992). It is possible that the Asian short-claw otter sprainting behaviour may convey a different message.

Relatively small differences were seen in the % fatty acid content when comparing the spraints of the different otter species. The lowest % fatty acid content was seen in the Canadian river otter spraint samples (% fatty acid mean = 3.61). The next highest were Asian short-claw spraint samples (% fatty acid mean = 4.42). Next highest were the Giant spraint samples (% fatty acid mean = 6.33) and the highest % fatty acid content was seen in European otter spraint samples (% fatty acid mean = 7.22). See Figure 6. All differences were statistically insignificant (see Table 4).

Chapter 6 demonstrated the percentage fatty acid content of both wild and captive European otter spraints to be much lower than that of the wild European otter anal sac secretion alone. This might be explained in terms of energetic cost. The wild anal sac secretion has high % fatty acid content, but only a very small amount is deposited along with faecal material in a spraint (although presumably in amounts large enough

to be effective for communication). This makes the most efficient use of the material available to the otter. Wild spraint samples tend to be smaller both in terms of percentage fatty acid content and weight than those from their captive counterparts. This might be explained by the availability of food sources. Energy conservation would be more important for wild otters having to forage for their own food, as opposed to captive otters that are provided with food regularly and reliably. Depositing secretion and faecal material in very small amounts might not be as important for a captive otter. In addition, captive otters by definition are confined to a relatively small area compared to the range of a wild otter. They are not able and have no need to use up huge amounts of energy – they do not need to forage for food or patrol large areas of territory in order to scent mark.

The differences in the % fatty acid content between the spraints of different species. (Unlike spraint weight) are not correlated with otter size, e.g, mean % fatty acid content of Giant otter spraints was lower (6.33) than that of the European otter (7.22) which is a substantially smaller animal (see Table 13)]. Fatty acid extraction from both anal sac secretion from the Canadian river otter, Asian short-claw otter and Giant otter would need to be analysed in order to to rule out or highlight any contribution directly made by diet the fatty acid. Additionally, spraint samples from wild Canadian river otter, Asian short-claw otter and Giant otter need to be analysed to compare weight and % fatty acid with those of their captive counterparts.

Comparisons of spraints from different otter species using Multi-Dimensional Scaling (MDS)

Identified fatty acids

The MDS analysis showed good separation of the otter species into groups for the absolute content data, indicating that the fatty acid composition of the 4 species is different (Figure 7). The MDS analysis for presence or absence data showed the best results of the analyses for the 'identified' fatty acids. This shows definite separation into species groupings (Figure 8). A significant overall difference was seen between all 4 sample groups, together with significant differences between each individual pair of otter samples.

In terms of similarity within species groups both palmitic and linolenic acid appear to play a significant role (see Table 9). Palmitic acid is the major contributor to similarity within species groups in European otter, Canadian river otter and Asian short-claw otter for the absolute content data. Linolenic acid is the major contributor to similarity within species groups in European otter, Canadian river otter and Asian short-claw otter for the presence or absence data. For the Giant otter the dominant fatty acid in terms of similarity within the species groups is erucic acid for the absolute content data and arachidic acid for the presence or absence data.

For the absolute content data, five fatty acids (palmitic, oleic, stearic, linoleic and palmitoleic) appeared in the top ten dominant fatty acids in terms of percentage similarity within species for every species (Table 1). Palmitic acid is present in similar amounts across all otter species. These fatty acids may be partly responsible for a typical generic otter odour in absolute terms that can be recognised by any of the species.

According to the SIMPER analysis, for the presence or absence data, four fatty acids appeared in the top ten dominant fatty acids in terms of percentage similarity within species for every species. However, these were all different acids to those for the absolute data (arachidic, arachidonic, behenic and tricosanoic). Therefore, the whole message conveying generic 'otter' may comprise an analogue element (absolute data) plus a digital element (presence or absence data).

Some of the fatty acids in the similarity top tens were unique to a particular species. For the absolute content data there were 2 for the European otter (arachidic and heptadecanoic), 1 for the Canadian river otter (linolenic acid) data, and 1 for the Giant otter (nervonic acid). For the presence or absence data only one species had unique fatty acids. There were 3 for the European otter (myristic, pentadecanoic and palmitic). These fatty acids may be partly responsible for conveying a typical odour for a particular species.

So, within the whole otter odour there may be a layer of odour conveying the 'generic otter' message and then a second layer conveying the 'otter species' message. It must be remembered though that this part of the analysis, not only just examined the top ten acids in terms of similarity within species, but also only included 'identified' fatty

acids. Therefore, the mechanisms conveying these possible odour messages could be much more complex.

In terms of dissimilarity between the species groups (see Table 10) for absolute content data, there was a variety in the dominant fatty acids responsible. These included lignoceric acid and nervonic acid. Similarly, for the presence or absence data there was a variety in the dominant fatty acids involved. These included linolenic acid and heneicosanoic acid.

Nervonic acid is present (in terms of absolute content) in varying amounts for each species. Analogue and digital coding are both present. Nervonic acid is seen in the highest amounts in the Giant otter samples, with relatively smaller amounts seen in the Canadian river otter samples (analogue coding). This is in contrast to the remaining species, which appear to have no nervonic acid present (an example of digital coding). Presence or absence data showed linolenic acid to be present in all species except the Giant otter (an example of digital coding).

For the absolute content data, two fatty acids (linolenic and heneicosanoic) appeared in the top ten dominant fatty acids in terms of percentage dissimilarity between species for every species comparison. For some of the comparisons the difference for these fatty acids is analogue (e.g., linolenic acid in European otter v Canadian river otter) and in some the difference is digital (e.g., heneicosanoic acid in European otter vs Asian short-claw otter). Some fatty acids only appeared in certain comparisons. There was 1 in the European otter v Giant otter comparison (tricosanoic – analogue) and 1 in the Asian short-claw otter v Giant otter comparison (arachidonic – analogue).

For the presence or absence data, just 1 fatty acid (heneicosanoic) appeared in the top ten dominant fatty acids in terms of percentage dissimilarity between species for every species comparison. For all of the comparisons the differences for this fatty acid were digital. Only one comparison included a unique fatty acid in the top ten, there was 1 in the European otter v Giant otter comparison (cis-13,16-docosadienoic – analogue).

From all this it can be seen that the fatty acid profile of each species is a complex mix of different acids, some define the similarities within species and some define the differences between species with both analogue and digital mechanisms. Messages of both ‘otter species’ and also ‘generic otter’ are possibly conveyed in this way.

All fatty acids

For the absolute content data, the MDS analysis showed good separation of the otter species into groups, indicating that the fatty acid composition of the 4 species is different (see Figure 9). This is a better result than when the 'identified' fatty acids were examined alone.

For the presence or absence data, the MDS analysis showed excellent separation into species groupings (Figure 10). These look the best results of all the data types (for either the 'identified' fatty acid data group or the 'all' fatty acid' data group).

In general adding further data has improved the separation of the different scent mark types, especially when looking at the data on a presence or absence basis. As with the identified fatty acid data alone, a significant overall difference was seen between all 4 sample groups, together with significant differences between each individual pair of otter samples.

The results from the SIMPER analysis (as with the 'identified' fatty acid data) showed that in terms of similarity within species groups for the absolute content data the result was very similar to when the 'identified' fatty acid data was examined alone. Palmitic acid was the major contributor to similarity within species groups in European otter, Canadian river otter and Asian short-claw otter for the absolute content data (see Table 6). For theGiant otter the dominant fatty acid in terms of similarity within the species groups is again erucic acid for the absolute content data. The most obvious difference in dominant fatty acids for the 'all' fatty acid data is with the presence or absence analysis. This time fatty acid 28 dominates for the European otter, Canadian river otter and Asian short-claw otter and fatty acid 27 for the Giant otter.

Palmitic acid (in terms of absolute content) is present in similar amounts in every species. Similarly, presence or absence data shows fatty acid 28 to be present in every species. These types of ubiquitous fatty acids may contribute to a generic otter odour, either by merely being present in every sample or being present in every sample in the same absolute amount.

According to the SIMPER analysis, for the absolute content data, three fatty acids (palmitic, oleic and stearic) appeared in the top ten dominant fatty acids in terms of percentage similarity within species for every species. These may be partly

responsible for a typical generic otter odour in absolute terms that can be recognised by any of the species.

Some of the fatty acids in the similarity top tens were unique to a particular species. For the absolute content data there were 3 for European otter (arachidic, heptadecanoic and lignoceric), 1 for Canadian river otter (linolenic) data, 1 for Asian short-claw otter (fatty acid 23) and 4 for Giant otter (nervonic, docosadienoic, fatty acid 33 and fatty acid 37). For the presence or absence data all species had unique fatty acids. There were 5 for European otter (fatty acid 38, tricosanoic, stearic, oleic and elaidic), 3 for Canadian river otter (heneicosanoic, fatty acid 18 and docosadienoic), 4 for Asian short-claw (fatty acid 30, fatty acid 36, fatty acid 17 and fatty acid 19) and 3 for Giant otter (fatty acid 27, fatty acid 29 and nervonic). These fatty acids may be partly responsible for conveying a typical odour for a particular species. It must be noted that many of these acids have changed upon addition of further data, emphasising the need for as much information as possible when examining complex olfactory mechanisms.

In terms of dissimilarity between the species groups (see Table 11) for absolute content data, there was variety in the dominant fatty acids. These included fatty acid 20, although fatty acid 23 appeared more than any other. Similarly, for the presence or absence data there was more variety in the dominant fatty acids involved. These included fatty acid 29 and fatty acid 30.

Fatty acid 20 in the absolute content data and fatty acid 29 in the presence or absence data are examples of digital coding. Fatty acid 20 appears in all species except for the European otter and fatty acid 29 appears in the Giant otter samples, but not in any other species.

Although the make up of the most dominant fatty acids (in terms of similarity within groups) has slightly changed with the addition of the unknown fatty acids, the results in terms of the MDS analyses have basically remained the same.

According to the SIMPER analysis, for the absolute content data, no fatty acids appeared in the top ten dominant fatty acids in terms of percentage dissimilarity between species for every species comparison. NB relatively few ‘identified’ fatty acids appear in the top ten dissimilarity compounds when ‘all’ fatty acid data is used in the analysis; unidentified fatty acids dominate. Some fatty acids only appeared in

certain comparisons. There were 5 in the European otter v Canadian river otter comparison (fatty acid 13 – digital, fatty acid 38 – digital, fatty acid 9 – digital, fatty acid 21 – digital and fatty acid 32 - analogue), 1 in the Asian short-claw otter v Canadian river otter comparison (fatty acid 17 - digital) and 4 in the Canadian river otter v Giant otter (fatty acid 8, fatty acid 12, linolelaidic and fatty acid 14 – all digital).

For the presence or absence data, no fatty acids appeared in the top ten dominant fatty acids in terms of percentage dissimilarity between species for every species comparison. There were 3 comparisons with unique compounds in the top ten. NB These were all comparisons involving European otter; 1 in the European otter v Canadian river otter comparison (erucic – analogue); 5 in the European otter v the Asian short-claw comparison (fatty acid 36, fatty acid 17, fatty acid 23, fatty acid 24 and fatty acid 2 – all digital); and 6 in the European otter v Giant otter comparison (fatty acid 29, fatty acid 35, fatty acid 37, fatty acid 18, fatty acid 22 and fatty acid 11 – all digital).

From all this it can be seen that the fatty acid profile of each species is an even more complex mix of different acids (when all fatty acids are included). Some define the similarities within species and some define the differences between species with both analogue and digital mechanisms. Messages of both 'otter species' and also 'generic otter' are possibly conveyed in this way.

A variety of fatty acids have been shown to be present in mustelid anal sac secretion. For example, low molecular weight fatty acids (common products of microbial action) have been identified. One possible function of odours produced by micro-organisms is to signal identity (the signal would need to be sufficiently constant over time to be representative of an individual). Olfactory encoding for individual identity has been observed in a range of other mammalian species. For example, the anal sac secretion of the Indian mongoose, *Herpestes auropunctatus* (Gorman, 1976).

In contrast, high molecular weight fatty acids have also been identified in the anal sac secretion of the European badger (Davies et al, 1988). Due to their less volatile nature, it has been suggested that high molecular weight fatty acids would be more suited to a long term signalling system and may have a role in territorial or home range marking (Davies et al, 1988; Hefetz et al, 1984; Wood et al, 2003).

The species in this study have been shown to contain high molecular weight long-chain acids of low volatility (in the range C_{12}-$C_{24.1}$). Volatile fatty acids were not detected. Therefore, these fatty acids may play a role in the maintenance of otter territories, both intra-specifically and also possibly inter-specifically. A generic otter odour and a typical species odour may be intrinsic to this function.

As discussed in Chapter 5, inter-specific communication will only be important for those animals that share habitats and perhaps only then if there is any overlap in the use of resources. In addition, such inter-specific communication through scent marking can also have disadvantages, e.g. it may alert potential predators to an animal's presence. Similarly, it could also have the potential to modify the behaviour of the animal's prey species. For example, water voles have been shown to avoid both mink and rat odour (Barreto & Macdonald, 1999).

CONCLUSIONS

- A measure of overlap between the peaks in the fatty acid profile of different species of mustelid was identified. Only one species had a 'species-specific' fatty acid.
- The presence of certain common chemicals could represent a typical 'otter' odour.
- A mix of 'digital' and 'analogue' coding seemed to be important in terms of identification of different species from their scent marks. Contribution of species-specific marker chemicals was limited.
- Expanding the number of fatty acids included in the analyses improved the separation between the species groups.

FUTURE WORK

- A repeat of this study using increased numbers of samples would need to be carried out in order to properly confirm the species-specific fatty acid blends.
- Analysis of anal sac secretion for the European otter was carried out in Chapter 4, but a similar study of the anal sac secretion in the other species would also be useful in terms of pinpointing contributions made to scent mark odour by diet.

- More of the unknown fatty acids could be confirmed using comparison with synthetic material. Especially as improved results were seen when using the whole fatty acid compliment.
- Behavioural studies to examine otters' responses to fatty acids would also be useful.

CHAPTER 8

GENERAL CONCLUSIONS AND FUTURE WORK

Discussion of research findings

This thesis was an examination of chemical communication in the European otter *Lutra lutra.* The opening chapter began with a thorough overview of scent marking in mustelids including, for example, subjects such as mustelid social organisation; the function of scent marking and odour sources. There was also an extensive, but concise summary of the body of work that covers the chemical analysis of mustelid scent mark material. The review continued by focussing on the European otter itself; it discussed the importance of the otter as an indicator of the health of the riparian habitat. It described in detail its decline in Britain since the late 1950s, its subsequent recovery and increase in distribution since the first national survey (1979/80- funded by the Nature Conservancy Council, the Royal Society for Nature Conservation (RSNC) and the Vincent Wildlife Trust) and the difficulties in finding reliable methods of surveying otter populations. Spraints, anal gland secretion and scent marking behaviour were highlighted and supplemented by an examination of the theories surrounding its function (seasonal spraint deposition and sexual behaviour; territoriality; resource marking and individual identity). Spraint ageing and time since deposition was also dealt with, before a final appraisal of previous studies concerning the elucidation of the chemical make-up of otter spraint material and anal gland secretion. This demonstrated the knowledge gap in this specific area of mammalian semiochemistry. Very few studies have been undertaken with limited identification of the volatile chemicals that comprise otter scent mark odour. Chapter 1 concludes with the list of aims and objectives set out at the very beginning of this research project and the first part of this final chapter will describe how these aims have been fulfilled.

Previous work, not only on the European otter (Gorman et al, 1978; Jenkins et al, 1981; Bradshaw et al, 2001) but also on other mustelid species (e.g., *M. vison*, Brinck et al, 1978; 1983 and *M. eversmanni admirati*, Zhang et al, 2002; 2003) generally utilised anal gland secretion sampled directly from the animal itself. Only one of the previous studies examined spraint odour profiles (Trowbridge, 1983), but no elucidation of the chemical constituents was carried out. This study mainly used freshly deposited spraint material for analysis. This comprised faecal material in addition to anal sac secretion. This approach

sought to obtain the whole picture of the deposited scent mark (as an otter would encounter it in the wild) and consequently the sampling process was as non-invasive as possible. This also meant that the sampling procedure was a matter of simply collecting a spraint as soon after deposition as possible.

Aim 1: To investigate the use of SPME in the extraction of otter spraint odour chemicals.

Chapter 2 of this thesis aimed to optimise SPME as a novel technique for use with otter spraints and anal sac secretion to be employed in conjunction with GC and GCMS. Initially, a chemical standard was used in order to compare direct injection with [SPME] headspace sampling (the differing volatilities of the chemicals in the headspace means that the concentrations detected will not necessarily match those found in the body of the sample itself).

Chapter 2 also pinpointed those SPME fibres that were most effective in extracting more volatiles overall from spraint material (red: 100µm polydimethylsiloxane, PDMS and blue: 65µm polydimethylsiloxane/divinylbenzene, PDMS/DVB) and optimised this technique. A specific exposure time (20mins), sample to headspace ratio (1:1), steady extraction temperature conditions, together with increases in the GC injector temperature, fibre desorption time and the introduction of an SPME inlet liner all contributed to greater precision and reproducibility in the SPME method. With the SPME method optimised for use with otter spraint material, a series of experiments were designed employing this sampling method.

Previous work, not only on otter scent mark material (Gorman et al, 1978; Jenkins et al, 1981; Bradshaw et al, 2001), but also on others within the mustelid family (see Chapter 1, Table 1 for summary) have all employed solvent extraction in order to identify those chemicals present. Part of the rationale behind this study is an attempt to extract only *relevant* volatiles from a spraint (i.e., those of interest to an otter). This is achieved by extracting the spraint volatiles in as similar a way as possible to the animal itself. This is crucial to our understanding of chemical communication in the otter. Solvent extraction results in extraction of chemicals from the body of the spraint. These may not be representative of the volatiles detected by a receiving otter from the surface of a scent

mark and this is undesirable. A headspace sampling technique would mimic to some extent the technique used by an otter by sampling volatiles from the air around the scent mark. SPME is a simple, relatively recent and successful technique (Belardi & Pawlisyzn, 1989; Arthur & Pawlisyzn, 1990) that allows sampling directly from the headspace of a (solid, liquid or gaseous) sample without the involvement of solvents. It is a technique utilised in a number of studies into mammalian scent marking material (subcaudal secretion of owl monkey, *Aotus nancymaae*, Macdonald et al, 2007; anal gland secretion, vaginal secretion and urine of giant panda, *Ailuropoda melanoleuca*, Hagey & Macdonald, 2003; anal gland secretion of the spotted hyena, *Crocuta crocuta*, Burgener et al, 2007), however, no studies have used SPME to investigate otter spraint volatiles.

Aim 2: To develop a technique for ageing spraints, possibly as a tool for monitoring purposes, by mapping the chemical changes in an otter spraint in the hours following deposition.

The first (SPME) study was featured in Chapter 3 and concerned spraint ageing. Few previous investigations into otter spraint ageing have been undertaken. This study examines how the chemical composition of otter spraint material changed in the days following deposition and how spraint material was affected by low temperature storage conditions and exposure to the environment. Here again, solvent extraction was compared to the SPME technique and it was concluded that more than one technique may be needed for a more rounded analysis of otter spraints in relation to the length of time exposed to the environment.

Otter spraint material was shown to have chemicals with 'opposite time dependence' which have the potential to be used for estimating the age of a spraint (a mechanism possibly used by scent marking animals, but also one which could be used by human surveyors). However, it was also shown that e.g., spraint location plus local weather conditions need to be taken into consideration as they can affect the rates of deterioration of otter spraint chemicals, particularly as a spraint gets older.

Aim 3: To determine whether spraint odour (extracted with SPME) can be used to identify individual European otters using a so-called otter fingerprint. Spraints from other otter species will also be analysed as a comparison.

The potential olfactory messages within *L. lutra* spraint material were investigated in Chapter 4. Initially the volatile headspace chemical constituents were sampled (using SPME), separated and identified (using GC/MS). Spraint samples from otters at different wildlife sanctuaries were obtained. The spraints were shown to contain >100 compounds (eg Tamar sanctuary: 104 / Bowes sanctuary: 121) that are typical of those found in the scent marks of other mustelid and non-mustelid species. Each chemical, represented by a peak in the chromatogram was given a value based on its TIC. Two categories of data were used for analysis, the raw or absolute data and simple presence or absence data. The analysis drew comparisons between 'sex' groups and between 'sanctuary' groups using a multivariate statistical technique, Multi- Dimensional Scaling (MDS). Spraint odour of otters from different sanctuaries showed significant differences (using ANOSIM) and it was thought that these differences may be diet related.

Do the captive *L. lutra* spraint odour profiles carry a message of identity for the individual otter? The low number of samples made it impossible to answer this question.

Within each sanctuary significant gender differences were also observed. When the sanctuary data was combined, results indicated that despite the apparent division within sex groups in the MDS, a significant difference between sexes was also present. The two main clusters of samples in the MDS related to differences in site groups but with further differences within each site group relating to sex. The statistical test (2 way ANOSIM) took into account the site groupings and indicated significant differences between sexes.

All distinctions seen (between sexes and sanctuaries) were accounted for by a mixture of digital and analogue coding. In addition, those chemicals responsible for the majority of the similarities and dissimilarities within and between the groups were ascertained (using SIMPER analysis).

The following chapter (5) complimented Chapter 4; it expanded the use of SPME to this time compare spraint material from different species of mustelid; the European otter (*L. lutra*); the Canadian river otter (*L. canadensis*); the Asian short-claw otter (*A. cinerea*)

and the European badger (*M. meles*). The presence of certain common chemicals across the species was demonstrated and these may represent a generic 'mustelid' or 'otter' odour. Most of the spraint odour profiles also possessed species-specific chemicals. MDS was again used for the analysis and ANOSIM demonstrated a significant differentiation in the odour profiles based on species; again a combination of digital and analogue elements was found.

Aim 4: To investigate the fatty acid content of both European otter (Lutra lutra) spraints and anal sac secretion in order to identify any individual or sexual differences. Again, spraints from other otter species will also be analysed as a comparison.

Chapter 6 investigated the intra-specific differences in the fatty acid profiles of *L. lutra* scent mark material (captive and wild spraints plus wild anal sac secretion). Within each scent mark type, fatty acids were extracted using solvents and identified, initially tentatively using MS software followed by confirmation through comparison with known chemicals standards. This resulted in positive identification of 23 fatty acids in captive spraints, 25 in wild spraints and 22 in wild anal sac secretion. All were of low volatility ($C_{10} - C_{24.1}$) and as such are suited to conveying an olfactory signal on a long term basis. The scent mark fatty acid percentage (dry weight) and spraint weight were ascertained and compared between scent mark types. Percentage fatty acid content of wild and captive *L. lutra* spraints was much lower than that of the wild anal sac secretion alone. Wild anal sac secretion had high percentage fatty acid content, but only a very small amount was deposited along with faecal material in a spraint (although presumably in amounts large enough to be effective for communication). Wild spraint samples were smaller both in terms of percentage fatty acid content and weight than those from their captive counterparts.

Do the captive *L. lutra* spraint fatty acid profiles carry a message of identity for the individual otter? Again, the low number of samples made it impossible to answer this question.

Some differentiation was seen in spraint fatty acid profiles based on sexual identity. . ANOSIM R values indicated no significant difference between the sexes for the absolute data with a weak significant difference between sexes for the presence absence data.

Results showed coding for this differentiation to be a combination of both analogue and digital elements (this was in common with previous chapters where headspace chemicals were analysed). For example, differences were detected in the absolute content of the five dominant fatty acids identified in *L. lutra* spraints (analogue coding). None of these differences were statistically significant. However, if *L. lutra* is able to detect these *possible* differences then this could provide a mechanism for distinguishing between the sexes.

MDS analysis showed a significant differentiation (confirmed using ANOSIM) in *L. lutra* scent marking material (captive spraint; wild spraint; wild anal sac secretion). Again, this was demonstrated to be due to a combination of digital and analogue coding, e.g. fatty acid 52 and fatty acid 46 contributed to digital coding in the dissimilarity between the scent mark groups and palmitic acid contributed to analogue coding.

Sulphur compounds are known to have attractant properties in other species. This study revealed the presence of 2 possible sulphur containing compounds derived from the *L. lutra* diet.

The penultimate chapter (7) expanded the fatty acid analysis used Chapter 6 to make inter-specific comparisons between a 4 species of otter, European, *L. lutra*; Canadian river, *L. canadensis*; Asian short claw, *A. cinerea*; and Giant, *P. brasiliensis*. A measure of overlap between the peaks in the fatty acid profile of the different species was identified. In only one species was a 'species-specific' fatty acid identified. The common chemicals (as in Chapter 5) may represent a generic 'otter' odour. MDS analysis demonstrated a definite differentiation in the fatty acid profiles based on species, again (using ANOSIM) this was found to be highly significant. As with the three previous chapters, a mix of 'digital' and 'analogue' coding seemed to be important in terms of identification of different species from their scent marks. Contribution of species-specific marker chemicals was limited.

Many studies of mammalian anal sac secretion have focussed on their fatty acid content (most posses either short or long chain fatty acids, or occasionally both). Although previous studies have indicated the presence of fatty acids in otter anal sac secretion, none have identified the specific acids involved or examined their role in scent marking.

Implications of the research findings in the context of previous study

In Chapter 1 the purpose of European otter (*Lutra lutra*) sprainting behaviour was explored. There were 4 categories:

Territoriality

Resource marking

Individual identity

Seasonal spraint deposition and sexual behaviour

In addition, Chapters 5 and 7 dealt with

Species identity

This section of the chapter will discuss the research findings of this thesis within the context of these 5 categories.

Territoriality

In Chapter 6, fatty acid extraction from *L. lutra* spraints revealed the exclusive presence of low volatility fatty acids. Davies et al. (1988) suggested that *M. meles* anal gland secretion, due to the presence of long chain fatty acids of low volatility would be well suited to long term signalling and may function in *M. meles* territoriality. This could also be true of the fatty acid content of the *L. lutra* scent mark samples.

Otters live in solitary or group territories with both aquatic and terrestrial elements (Kruuk et al, 1998). In coastal *and* freshwater habitat *L. lutra* show similarities in their social organisation with male ranges overlapping 2 or more female ranges (*coastal*: Erlinge, 1968; Kruuk, 1995; *freshwater*: Kruuk et al, 1993; Durbin, 1993). If sprainting functioned in territoriality, then concentration of spraints at range borders might be expected (Gorman & Mills, 1984; Gorman & Trowbridge, 1989), however a study by Kruuk (1992) found this not to be the case for *L. lutra.* This contrasts with other carnivorous mammals, e.g., badgers, where boundary scent marking is extensive (Kruuk, 1972, 1978a, 1989; Mills, 1990). *L. lutra* live alongside and forage in rivers so their ranges are linear in shape. Similarly, those living and foraging next to the coastline also only use a relatively narrow strip of water and land (Kruuk, 1995). Therefore, an otter's

territory is less defensible economically than, e.g., a badger and this is reflected in the scent marking strategy. Instead of boundary marking, otters limit their scent marks to particular areas within their territory.

The 'match hypothesis' (Gosling, 1982) states that matching an individual's odour with that of the majority of the scent marks within a territory, an intruder is able to identify it as the resident. The intruder can then assess the resident's investment in the territory, and will usually withdraw from any potential conflict. In theory, this prevents escalation of the encounter into a full-scale fight.

Kruuk (1995) contends that the 'match hypothesis' would probably not apply to *L. lutra*. He qualifies this by explaining that, unlike many other carnivores, otters appear not to aggressively defend territories, and that face-to-face meetings are rare. However, in autopsies on road kill otters from southern and south-west England, many animals (including mature males) have been seen to have injuries from violent encounters with other otters (Simpson, 2006).

If *L. lutra* does use sprainting as a means of territorial defence then they may have the ability to differentiate between spraints of known and unknown individuals, or at least to separate its own smell from those of others. Also, if the match hypothesis *did* apply to *L. lutra*, then they would need to be able to 'match' the odour of territorial spraints to that of their depositor. Research investigating the ability of *L. lutra* to distinguish between familiar and unfamiliar spraints has produced conflicting results. Studies by Trowbridge (1980; 1983) and Rozhnov & Rogoschik (1994) showed that otters did indeed have this ability; however a study by Durbin (1989) failed to show a similar result.

In Chapter 6, no differentiation in the spraint fatty acid profile was seen between *L. lutra* individuals. Similarly, in Chapter 4, the spraint odour profiles (extracted via SPME) also showed no differentiation between individuals.

When an intruding otter finds a territorial spraint it is important for that animal not only to be able to tell that the spraint comes from another individual, but also *when* the spraint was deposited. This would allow an assessment to be made as to the current situation in terms of ownership or occupation of a territory. Chapter 3 investigated possible mechanisms, which might be used for ageing of deposited *L. lutra* scent marks. Otter

spraint material was found to contain chemicals with opposite time dependence, which could form the basis of a mechanism for pinpointing the age of a spraint. This could have (obviously a useful tool both for the animals themselves, but also for human surveyors). Additionally, it was demonstrated that environmental conditions (location, temperature, local weather) can have an effect on the rates of spraint chemical production and deterioration. This will affect accuracy of spraint age estimation in the field.

If otter spraints are used in territoriality, then they need to be stable in the environment for periods of time that (at least) match the frequency a scent-mark will be encountered (Albone & Perry, 1976; Albone et al.1977; Preti et al. 1977).

Resource marking

The use of a resource marking strategy may require mechanisms similar to those explored in the previous section on territoriality.

1) a relatively long term signal might be required (Chapter 6 revealed the exclusive presence of long chain fatty acids of low volatility which could be used for such a purpose).

2) if *L. lutra* uses sprainting in order to mark important resources then they may have the ability to differentiate between spraints of known and unknown individuals, or at least to separate their own smell from those of others. As previously mentioned Chapter 6 showed no differentiation in the spraint fatty acid profile between *L. lutra* individuals. This result was repeated in Chapter 4 (this time with spraint odour profiles extracted via SPME). However, this was an unreliable result given the low numbers of samples obtained and further work with an increased number of samples would needed to investigate more fully any individual differences. However, if the resource marking strategy was non-confrontational means of resource partitioning then individuals would rarely meet and the ability to 'match' spraint odour to that of its depositor would be less important (than for instance in the territorial 'match hypothesis').

3) In the previous section the role of diet in differentiating individuals and their status was discussed in terms of territoriality. In Chapter 6, it was speculated that

dietary intake may partly explain the differences in the scent mark groups. The diet of wild *L. lutra* can fluctuate (seasonally and with location) possibly affecting spraint odour profile. It is possible therefore that faecal odour in spraints could indicate individuals feeding in a particular area and this might contribute to the resource marking strategy.

4) If a resource marking strategy is to function, then an otter finding a scent mark must not only to be able to tell that the spraint comes from another individual, but also *when* it was deposited. This would allow an assessment of the situation in terms of current foraging activity in the immediate area. In Chapter 3 it was shown that spraint material contains chemicals with opposite time dependence. These could provide a mechanism for estimating the age of a spraint. Environmental conditions (location, temperature, local weather) were demonstrated to affect the rates of spraint chemical production and deterioration and consequently the accuracy of spraint age estimation in the field. If otter spraints are used for resource marking, they need to be stable in the environment for periods of time that (at least) match the frequency a scent-mark will be encountered (Albone & Perry, 1976; Albone et al, 1977; Preti et al, 1977). Otter spraint odour was stable (to the human nose) for somewhere between 8 and 16 days (personal observation); but how long does a spraint persist as a scent mark detectable by the olfactory apparatus of an otter? Replenishment of a spraint before the previous one has become ineffective would produce a permanent 'resource marking' olfactory feature .

Kruuk (1992) suggests an alternative explanation for the role of sprainting in Shetland otter populations: as signals to other otters that a particular stretch of water, is being, or as already been, utilised. Otters have the ability to recognise individuals from spraint odour (Gorman & Trowbridge, 1989) and this would be needed for such a strategy to work (Kruuk, 1995). Otters utilise the same feeding sites, holts or freshwater washing pools over and over again and advertising this usage has certain advantages. Otters may benefit from moving away from feeding sites in use by other individuals, and finding another location free from competition. Resident otters also benefit as this strategy allows them to

forage without competition. Otters may have developed a non-confrontational system, which allows partitioning of resource utilisation within their territory (Kruuk, 1995).

Other studies have supported this explanation of the role of sprainting. Available fish biomass has been positively correlated with sprainting frequency (Prenda & Granado-Lorencio, 1996). In addition, a positive correlation has been found between the number of suitable sites for fish and sprainting intensity (spraints/km).

Durbin (1989) found that otters overmarked spraints whether or nor they were from familiar individuals. A similar conclusion to that of Kruuk (1992) was reached, i.e., otters may not be classically territorial with sprainting behaviour perhaps simply advertising an individual's presence in order to provide personal foraging space.

Individual identity

As mentioned in previous sections Chapter 6 showed no differentiation in the spraint fatty acid profile between *L. lutra* individuals; a result repeated in Chapter 4 (this time with spraint odour profiles extracted via SPME).

Sprainting behaviour has been linked with home range advertisement, territorial marking (Erlinge, 1967b, 1968; Kruuk & Hewson, 1978; Macdonald 1980) and resource marking (Kruuk, 1995). Otters may therefore be able to differentiate between spraints of known and unknown individuals, or at least to distinguish their own odour from those of others.

There are three ways that individual (or indeed, sexual) identity might be conveyed:

1. *Digital* coding - unique individual scent compounds in each of an individual's spraints. For example, possibly similar to the sex specific scent components in the anal gland secretion of the African mongoose, *Herpestes ichneumon*. (Hefetz et al, 1984).
2. *Analogue* coding - spraints of every individual could comprise the same scent components with their relative proportions providing an individual odour 'fingerprint'.
3. A combination of both *Digital* and *Analogue* coding.

Sun & Müller-Schwarze (1998, 1999) first used the terms *digital* coding (presence or absence of compounds) and *analogue* coding (relative abundance of shared compounds) in their studies of anal sac secretion secretion in the North American beaver, *Castor canadensis*.

Work undertaken to show whether otters actually have the *ability* to discriminate between spraints from different individuals has produced conflicting results.

- Training associated with rewards was used to study the ability of 2 captive otters (1 male / 1 female) to discriminate between spraints in 2 categories (Trowbridge, 1980, 1983):
    - *individual's own spraints* vs *spraints from other individuals*
    - *familiar spraints* vs *unfamiliar spraints*

  The trained male otter was successful in both these categories.
- Durbin (1989) investigated the ability of wild otters to distinguish spraints using the same 2 categories used above; *overmarking* was used as an indirect measure of response. In contrast to the Trowbridge study, there was no discrimination between spraints within the 2 categories.
- Rozhnov & Rogoschik (1994) showed that captive otters were able to distinguish between their own spraints and those of other otter individuals, confirming the Trowbridge result.

These results imply that the wild otters are able to discriminate between the odours of different spraints, but that the oversprainting response is not an indication of this. Durbin's study showed that strange and familiar spraints were both overmarked by the resident otter indicating that perhaps the purpose of sprainting was not territorial. However if, sprainting was used in order to advertise the use of resources (Kruuk, 1992) and maintain spacing between individuals regardless of their familiarity then this indiscriminate overmarking behaviour would be expected.

Seasonal spraint deposition and sexual behaviour

In Chapter 6, differentiation in the spraint fatty acid profile was seen between *L. lutra* males and females. Similarly, in Chapter 4, the (within sanctuary group) spraint odour profiles (extracted via SPME) also showed differentiation between males and females. This in itself implies that there may be more than one mechanism for the identification of sex via scent marks. Otters may be able to utilise one or other of these or maybe even both.

Chapter 4 and Chapter 6 (respectively) show possible differences in the odour and fatty acid profile of captive *L. lutra* spraint material based on gender. But why would a deposited spraint require separate mechanisms to relay the same message? The chemicals extracted in the odour via SPME include a variety of volatile compounds that may produce a transient, short-term message and in contrast the low volatility fatty acids extracted (solvent) from the body of the spraint are more suited to a long term signal. It may be then that the 2 identical messages are required in order to allow the signal to last as long as possible in the environment following deposition. Chapter 3 demonstrates a possible mechanism for estimating spraint age and although this involves chemicals with opposite time dependence, there must also be chemicals that are more stable if the signal is to persist long term.

Past research has looked at differences between male and female otters purely in terms of differences in the *numbers* of spraints produced. For example, Erlinge (1968) contended that sprainting might be expected to be most intense during the breeding season. This type of seasonal fluctuation was seen in a study by Conroy & French (1987) with less spraints found in winter than summer. Shetland otter populations reproduce seasonally (Kruuk et al, 1987; Kruuk 1995) and so there may be a link between the two.

Seasonal patterns of spraint deposition have also been seen in freshwater habitat (Macdonald & Mason, 1987; Ottino & Giller, 2000); however, here reproduction can take place at any point in the year (Erlinge, 1967b; Harris, 1968). Despite this, in Sweden, for example, otters tend to mate in late winter or early spring. Sprainting activity during this period was high, but not exceptionally high compared with other periods (Erlinge, 1968). This may place suggestions of a link between sprainting and reproduction in doubt.

It may be the case that numbers of spraints may not have significance in terms of sexual behaviour and that the olfactory messages within the spraint may hold more importance. For example in freshwater habitat, where reproduction can take place at any time of the year, an *ever-present* male or female olfactory message might be necessary element within the otter spraint odour. This would perhaps enable a male territory holder to locate the females within his territory in order to mate, regardless of the time of year. This might also explain the secrecy displayed by females around the birth of their cubs. Males have been known to carry out infanticide and remains of small *L. lutra* cubs have been found in the stomach of a *L. lutra* male (Simpson & Coxon, 2000). Avoidance of infanticidal males is likely to be the main biological advantage of the secretive behaviour shown by *L. lutra* females with cubs (Kruuk, 2006). Natal holts are notoriously difficult to locate, they are different from 'normal' holts in that they appear to be unlikely places for an *L. lutra* to be found (Kruuk, 2006). Following a foraging bout, a female with cubs will take extreme care not to be seen slipping back into the natal holt, hardly showing herself. Female *L. lutra* can also show aggression towards unknown males if they venture too close to young cubs and this is thought to be part of a strategy of protection (Kruuk, 2006). In addition, females have been reported to spraint less after the birth of their young with the lowest number of signs observed for a month after the birth of a cub, when the otters remained in and around the natal holt (Prigioni et al, 1995).

A constant female message in spraint odour would mean it would be difficult to 'hide' from male otters in order to protect their young from infanticide and the only options would be those discussed above i.e., aggression towards males, a reduction in sprainting frequency or a change to more secretive behaviour plus retreat to the natal holt.

Species identity

Chapter 5 and Chapter 7 investigated possible inter-specific differences in spraint fatty acid profile and spraint odour profiles - extracted via SPME (respectively). In both these chapters, each species examined could be distinguished from their scent marks (with the human nose) suggesting the possibility that one of the basic functions of spraint odour may be to signal species identity. In Chapter 7, the individual otters sampled could be classified to species through digital and analogue coding in their fatty acid profiles,

although the contribution of species-specific marker chemicals was limited. Along with these inter-specific differences in the fatty acid profiles, some overlap between the different species was identified. The presence of certain common chemicals could represent a general 'otter' odour. Chapter 5 produced very similar results to those seen in Chapter 7, but this time spraint odour profiles (extracted with SPME) were used in the analysis. Again, digital and analogue coding could be used in order to classify otters at the species level and the contribution of species-specific marker chemicals was limited. Spraint odour overlap between the different species was identified and the presence of common chemicals could represent a general 'otter' or this time 'mustelid' odour.

Mustelids have evolved into specialized species able to exploit a variety of environments (Neal & Cheeseman, 1996). Otters are found in most continents and overlap in the range of different species is usually observed along with major differences in diet or habitat (Kruuk et al, 1994). In South-East Asia 3 otter species occur sympatrically, Asian short-clawed (*Aonyx cinerea*), European (*Lutra lutra*), and smooth otter (*Lutra perspicillata*). Although there are differences in their use of habitat and in their diet, there is also some overlap (Kruuk et al, 1994). Sprainting behaviour in *L. lutra* has been linked with home range advertisement, territorial marking (Erlinge, 1967b, 1968; Kruuk & Hewson, 1978; Macdonald 1980) and resource marking (Kruuk, 1995). If this, *intra*-specific communication through scent marking is to work effectively then an animal must be able to at least distinguish its own odour from that of another individual. This basic premise must also hold true for effective *inter*-specific communication (Brinck et al, 1983).

Given that otters can be distinguished through both their spraint fatty acid profile and their spraint odour profile (extracted with SPME), it may be that there is more than one mechanism for the demarcation of sex.

If otters were able to detect these differences then this could provide a mechanism intra-specific communication.

The possible influence of diet

Differences in dietary intake may play a role in the differentiation of spraints and scent mark material.

The status of an individual with in a territory may be partly indicated through diet. In Chapter 6, there were differences between the scent mark groups (fatty acid profiles of the wild otter sample group was found to be distinct from that of their captive counterparts) and in Chapter 4, there were differences seen in the spraint odour profiles of otters from different sanctuaries – this could all be partially due to diet. Also, in Chapter 6, differentiation in the spraint fatty acid profile was seen between *L. lutra* males and females. Similarly, in Chapter 4, the (within sanctuary group) spraint odour profiles (extracted via SPME) also showed differentiation between males and females. Again, diet was suggested as a partial explanation. Finally, the differentiation observed between species in both chapters (5 and 7) has, in part, been attributed to dietary influences

Wild *L. lutra* diet is subject to seasonal and locational changes and this may have an effect on the spraint odour profile, which in turn might help to identify animals foraging in a particular locality. Fishstocks, both within and between river catchments in the UK differ significantly (Nunn, 2007). As a consequence, faecal odour in spraints could indicate status; territory holders with access to superior feeding grounds may be partially identified by intruders via the intensity of the food odour in their scent marks.

L. lutra with well-developed hunting skills acquired through experience will have improved hunting success (Watson, 1978; Shannon, 1991). Less experienced individuals may feed on prey that is easier to catch, but are less rewarding. This was not supported by the results of the Heggberget & Moseid (1994) study on Norwegian *L. lutra*. Sub-adult otter diet was found to be similar to that of adult *L. lutra*. It was inferred that these Norwegian *L. lutra* are not limited by food resources. If food resources are patchy, social rank may control the type and quality of prey individual *L. lutra* has access to (Heggberget & Moseid, 1994).

Diet may not only play a role in differentiating sexual identity, but also in mate preference. A study of the meadow vole, *Microtus pennsylvanicus* sought to investigate

the attractiveness of odours produced (from the anogenital area, urine and faeces) when the animals were fed diets that differed in protein content (Ferkin et al, 2002). Male and female subjects preferred odour from consumers of a high-protein diet. This suggests a mate selection mechanism based on odour preferences influenced by the diet of the potential partner. Animals in wild situations compete for territories with higher-quality food resources and therefore choosing a mate with a high quality diet would mean choosing a mate of higher quality. A similar mechanism to that of *M. pennsylvanicus* may be operating for *L. lutra.*

Sex and age have been shown to affect the size and type of prey foraged by *L. lutra* on Shetland (Kruuk & Moorhouse, 1990). On average, male *L. lutra* are 45% heavier than females (Chanin, 1985) and take either more or larger prey due to their bigger energy needs. Female *L. lutra* are known to catch large prey for their cubs and then eat smaller for prey themselves whilst in the water (Kruuk et al, 1987; Heggberget & Moseid, 1994). This may mean that males and females are consuming different species of fish. These differences in diet taken by males and females may play a crucial role in distinguishing between the sexes.

All the different species of mustelid examined consumed slightly different diets (the most obvious comparison being that between the wild otters and their captive counterparts) and this again was used to partially account for the inter-specific differences in spraint odour profiles. Different otter species occurring sympatrically have been demonstrated to exhibit some differences in their dietary preferences (Kruuk et al, 1994).

Significance of research findings

Most of the research carried out in this thesis had never been undertaken by previous researchers and so it presents a variety of novel pieces of information, which are of interest. For example:

- The development and use of SPME to sample volatile chemicals in the headspace of otter spraint material is a new use of this technique. This has enabled the investigation, analysis and comparison of spraint headspace volatiles, not only within *L. lutra*, but also within and between other mustelid species.
- The possible ageing mechanism discovered in the chemical changes following spraint deposition could have implications for future studies involving otter monitoring and conservation; with the possibility of providing a temporal map of otter movements in the field.
- Extraction, identification and analysis of fatty acid profiles from *L. lutra* and other otter species has never been carried out before by previous researchers. Also, comparison between whole spraint material (includes faecal material and anal sac secretion) and anal sac secretion alone is a novel investigation.
- Research into a bacterial presence within the anal sacs of *L. lutra* has also never been carried out before. And although this part of the thesis was supplementary to the main body of work it is nonetheless an interesting aspect.

Due to the novel nature of many aspects of this research, understanding of otter chemical communication has been enhanced.

Limitations of the findings and recommendations for future research

Whilst the findings of this research project are presented here with confidence, it must at the same time be recognised that the project is a study into only a small aspect of a hugely complex subject area. Even large scale, comprehensive studies have limitations due to, for example, methodological restrictions or practical realities. The following documents the limitations of this research project in conjunction with some recommendations for future research.

- All the studies carried out would benefit from being repeated with a greater number of samples in order to confirm and improve the results.
- The study on spraint ageing (Chapter 3) should be repeated using GC-MS analysis rather than GC alone in order to identify those chemicals with opposite time dependence.
- In general, more of the compounds tentatively identified in this thesis should be confirmed using comparison with synthetic material. Especially as improved results were seen, for example, when using the whole fatty acid compliment.
- Behavioural work with both captive and wild otters should be carried out in order to test their ability to distinguish between spraints from different individuals and between spraints of different ages. This could be carried out through training animals using a system of rewards or through using an indirect measure, e.g., an overmarking response.
- Behavioural work with both captive and wild otters should also be carried out in order to test their ability to distinguish between spraints from different genders. Attempting this with spraints of different ages may shed more light on the possible existence of 2 mechanisms (short and long term) for the signalling of gender.
- Further behavioural studies comparing captive otters to their wild counterparts could be undertaken in order to verify the results seen in Chapter 8.
- In addition, 'artificial' spraints could be produced using, e.g., chemicals from spraint odour or from the spraint fatty acid profile.

- The possibility of certain chemicals or combinations of chemicals being attractants (e.g., food odour chemicals or sulphur-containing compounds) or deterrents (e.g., chemicals from wolf scat odour) could be investigated, again through behavioural studies.
- Anal sac secretion analysis of was carried out in Chapter 6, but a similar study of the anal sac secretion in the other species would also be useful in terms of pinpointing contributions made to scent mark odour by diet.
- Perhaps urine could be examined as a source of male / female differences.
- SPME has been used to demonstrate inter-individual variation in the volatile compounds found in scent marks of spotted hyenas (Hofer et al, 2001). Further development in the method is necessary to investigate its sensitivity to individual differences between scent marks within otter and other mustelid species, with a view to its possible use as a monitoring tool in the field.
- Further investigation of the possible biofilm communities (seen in Chapter 8) with epifluorescence microscopy using a DNA stain (e.g. DAPI, sybr gold, or syto9) to check that these structures are biological in nature.
- Culture of the organisms to investigate in more detail whether they produce metabolites involved in mammalian behaviours.

It is evident that little is still known about the chemical communication in the European otter, *L. lutra*. Unraveling scent-marking behavior is fundamental to our understanding of this member of the diverse and highly specialized family of Carnivores, the mustelids.

First hand observation of these secretive and often nocturnal creatures is difficult to obtain and so scent-marking provides an opportunity for indirect population assessment. To this end information regarding typical patterns of otter scent-marking behavior in undisturbed populations as well as an understanding of the mechanisms required for these systems to work is essential. Knowledge of this kind would be useful for management, not only of the otter, but also of other (scent marking) species and of many aspects of their shared environment.

- With the appropriate information, established otter populations could be monitored and the success of conservation measures such as habitat modification and introduction / translocation of new individuals could be assessed by comparison with normal scent-marking activity.
- Use of attractants and/or deterrents, developed from our understanding of otter odours might allow manipulation of otter behavior. This would be particularly useful in areas where otter populations are affected by road kill (identified as being an important contributor to mortality not only in the otter, but also in the European badger in Britain).
- Much recent research into mustelid scent marking behavior has been driven by mustelids indirect effect on human activities, e.g., their status as reservoirs for domestic animal disease (badgers and cattle - bovine TB / stoats and ruminants - Johne's disease) or their role in the predation of game birds (stoats and weasels). A greater understanding of scent-marking behavior would lead to more effective management plans.
- Mustelid scent mark information would also be useful for managing other species, e.g., employing mustelid scent in order to deter other species from plant damage and the development of lures in an attempt to reduce the predatory threat of mustelids on other species by trapping (e.g., stoats threat to native hole-nesting and flightless birds in New Zealand's mainland forests).

These issues will hopefully continue to generate funds for future research, increasing knowledge of otter scent marking behaviour, knowledge that will help to ensure its continuing survival.

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