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Population genetics and PBDE analysis of English and Welsh otters

Integrated catchment science programme
Science report: SC040024/SR1

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Author(s):
Angela Pountney, Jamie R. Stevens, Tim Sykes, Charles R. Tyler

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Environment Agency's Project Manager:
Tim Sykes, Science Department

Collaborator(s):
University of Exeter Biosciences department, Biotechnology and Biological Sciences Research Council UK (BBSRC), Hampshire and Isle of Wight Wildlife Trust, Cornwall Wildlife Trust

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Executive summary

Otter populations declined drastically across many areas of England and Wales during the 1960s to 1980s. The main cause of this decline is thought to have been high concentrations of organic pollutants, in particular PCBs and dieldrin. This report investigates the health of present day otter populations in England and Wales and, in particular, populations in southwest England. The research focuses on otter numbers and the genetic diversity of populations. It also investigates a possible new threat from organic pollutants, polybrominated diphenyl ethers (PBDEs).

In southwest England, research focused on two catchments, the River Camel in Cornwall and the River Itchen in Hampshire. A non-invasive, spraint genotyping study of the otter population inhabiting the Camel, revealed that a minimum of 16 otters used the river during the two consecutive seasons of study (October 2005 - May 2006 and October 2006 - June 2007). The research also provided insight into the ranges and genetic relationships of otters using the river. A genotyping study was also carried out on the otter population on the Itchen. This population declined drastically in the 1950s and 1960s, to just a few isolated individuals, before being supplemented with otters released as part of a captive breeding programme. Microsatellite genotyping of tissue samples showed the Itchen otter population to be relatively diverse, indicating a successful population recovery Additional analysis of genetic haplotypes indicated that captive bred otters have successfully interbred with wild otters, contributing to the genetic profile of the current Itchen population.

In a second strand to the project, PBDEs were added to the existing list of organic pollutants detected in otter livers. The concentrations of PBDEs found in otters rival the high concentrations observed in many marine mammal species and are approaching the concentrations of PCBs and DDTs already reported in otters. The profile of PBDE congeners found in otters shows that BDE-47 is by far the most concentrated BDE congener, following the trend found in many aquatic environmental samples. Congeners BDE-99 and -100 are also found at significant concentrations. Otters contain relatively high concentrations of the congeners BDE-153 and BDE-209, a trend more typical of terrestrial top predators.

In summary, the otter populations studied in southwest England appear to be recovering well. Genetic diversity of the populations appears to be recovering and levels of diversity observed in the Camel and in the Itchen, a river known to have received substantial input from captive bred animals, are similar. The extant otter population of the Itchen shows evidence of genetic input from releases of captive bred animals. High concentrations of PBDEs have been detected in a range of otter tissues; what effect these levels may be having upon the species is unknown.

Acknowledgements

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Spraint sample collections were organised by Graham Roberts from the Hampshire and Isle of Wight Wildlife Trust and Kate Stokes from Cornwall Wildlife Trust. Collections were carried out by a team of volunteers without whom the spraint study would not have been possible. Thanks to all the volunteers involved and, in particular, we would like to thank Robert Hurrell, Tyson Jackson, Jon Evans and Chris Macham, all of whom we know put in particularly long hours on the riverbank.

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1 Introduction

1.1 Population decline and recovery

During the 1950s and 1960s otter populations in Britain and many other Western European countries declined drastically. Populations across mainland England declined to such an extent that by the late 1970s only 6% of sites deemed suitable for otter habitation showed any signs of otter activity, according to a national survey (Lenton *et al.*, 1980; Crawford, 2003). This decline was not uniform, populations in the south west of England remained relatively healthy (23.5% occupancy in the 1977-79 survey), while signs of otter activity in the north and east of the country became extremely rare, to the point that, in some regions, otters could have been considered extinct (Crawford, 2003). The south west of England, Scotland and Wales all suffered less significant declines than other areas and remained as otter strongholds during the recovery of British otter populations (Dallas *et al.*, 1999; Crawford, 2003).

Possible causes of the decline in otter numbers include direct persecution, habitat destruction, fish stock declines and the increasing pressures of road traffic. Although each of these factors is likely to have contributed to the overall decline in otters, individually they are unlikely to have caused the severe declines observed over a relatively short period in time. Organic pollutants, such as polychlorinated biphenyls (PCBs) and other organochlorines (OCs), are thought to be the most likely cause of the rapid and major decline in otter numbers. In particular, the chemical compound dieldrin (HEOD), used as an insecticide in crop sprays and sheep dips, was brought into widespread use in the years immediately prior to, and during the decline, and is thought to have been a major contributor to the severe reduction in otter numbers (Chanin and Jefferies, 1978).

Over recent decades otter numbers have been slowly recovering (Crawford, 2003). In many areas this recovery has been as a result of natural population expansion and recovery. However, in several areas of England otter numbers have been bolstered by the release of captive bred individuals. These releases may well have saved several of the English otter populations from extinction, but questions have been raised over the genetic input from released otters, as many were bred from otters of unknown origin (referred to by breeders as ‘B line’ otters).

1.2 Background to this study

In order to monitor what is going on in wild populations and to assess whether further intervention is needed to prevent future declines, it is essential to gain as much information about wild populations as possible. Many previous surveys have relied on the sighting and recording of otter signs, such as spraint or tracks, to monitor for otter presence and absence. This technique is useful for assessing large scale habitat occupancy, but provides less information on fine scale details of otter movements and precise otter numbers. Genetic typing (genotyping) of spraint samples provides an effective way of counting the number of otters present in a survey area. Genetic

information can also be used to provide an insight into the genetic history of a population.

During the declines of the 1950s and 1960s the number of otters inhabiting the River Itchen in Hampshire is thought to have dropped to just a handful of individuals (Crawford, 2003). The surrounding river systems are thought to have become virtually devoid of otters during this period, effectively isolating the River Itchen otter population. During the early 1990s at least four captive bred otters were released onto the River Itchen. In addition, there is evidence to suggest that the otter population in the south west of England is expanding in range and that the leading edge of this expansion may now have reached the River Itchen catchment. Taken together, these factors suggest that the River Itchen otter population underwent a severe bottlenecking event, followed by injections of genetic material from captive bred releases and the expansion of neighbouring populations. One of the aims of this study is to assess the extent to which the River Itchen otter population has been affected by these historic population alterations.

In contrast, the otter population on the River Camel in Cornwall remained relatively healthy during the declines (Crawford, 2003). The fact that the river is situated in an area of otter stronghold is one of the primary reasons for the River Camel catchment being designated a Special Area of Conservation (SAC). The river is situated in the heart of south west England, in an area where otter populations suffered a much lower decline in their numbers. This ensured that the River Camel population maintained healthy population numbers and maintained migration routes to and from surrounding river populations; a process that would have helped to maintain the genetic diversity of the population. In this study the River Camel otter population was used, not only as a comparative study population to the River Itchen population, but also as an example of a relatively healthy river population that could be used to study population dynamics on a local scale using the spraint genotyping techniques.

In addition to studying population dynamics, through the use of population genetic techniques, a further aim of this study was to investigate contamination levels from endocrine disrupting chemicals (EDCs) in otters. Consequently, we added a new suite of compounds, polybrominated diphenyl ethers (PBDEs), to the list of compounds already analysed by the Environment Agency otter project (Simpson, 1998, 2007; Chadwick, 2007). Over the years PBDEs have been used as flame retardants and added to a wide variety of materials, including electrics/electronics, textiles (not clothing), building materials and household appliances (Hooper *et al.*, 2004; BSEF, 2006). Manufacture and use of PBDE industrial mixtures has recently been banned within the EU (BSEF, 2008). However, the compounds' heavy historic usage and environmental persistence means that, like the similarly structured PCBs, PBDEs are likely to contaminate environments for many years to come. As a top semi-aquatic predator the otter is a prime target for contaminants, such as PBDEs, which are likely to build up and biomagnify up the aquatic food chain. PBDEs are known to have endocrine disrupting and neurotoxic properties, which makes their monitoring in sensitive species, such as the otter, all the more important.

1.3 Project objectives

- Improve spraint genotyping techniques
- Improve knowledge about the status and health of the River Itchen and River Camel otter populations
- Measure concentrations of PBDEs in otters from across England and Wales
- Compare concentrations of PBDEs to concentrations of PCBs and OCs in otters

2 Genetic analysis

2.1 Sample collection

Tissue samples for genetic analysis were collected as part of *post mortem* examinations of otter carcasses, a process which is described in more detail in section 3.1 *Liver tissue collection*. The majority of samples collected specifically for genetic analysis consisted of kidney or liver tissue stored either frozen or in 95% ethanol.

Spraint samples for microsatellite genotype analysis were collected from the River Itchen catchment in Hampshire and the River Camel catchment in Cornwall. These collections were carried out by teams of volunteers, trained and co-ordinated by Graham Roberts from Hampshire and Isle of White Wildlife Trust and Kate Stokes from Cornwall Wildlife Trust. The collections were carried out in a similar manner to those described by Coxon *et al.* (1999), with volunteers visiting known sprainting sites that had been identified during the initial stages of the study.

Volunteers were encouraged to collect once a month over a dedicated weekend, although volunteers also collected *ad lib* during the study period. Spraint samples were transferred directly into a tube of IMS using natural implements such as twigs and leaves, so as not to transfer contamination from one collected sample to the next. Tubes were labelled with relevant information to enable the sample to be tracked (largely left to the discretion of the volunteers). Although records were not consistently kept, volunteers were encouraged to fill in data sheets regarding the samples collected – an example of which can be seen in Figure 2.1. This provided dates and locations for individual samples and information about the field conditions the spraint samples were subjected to. This allowed us to predict what effects these conditions might have on the success of DNA extractions.

The surface of the lining of the mammalian gut is similar to the surface of the skin in that it regularly sheds dead and dying cells and replaces them with new ones. It is the DNA from these shed cells that was targeted in the spraint material analysed in this study. Once these cells have been incorporated into the spraint they are no longer part of a living tissue and as such lose the protection of the defence and DNA repair systems present in living tissue (Lindahl, 1993; Handt *et al.*, 1994). Once the spraint has been excreted from the body it becomes a target for bacterial action, which breaks down any cellular material not fully digested during passage through the otters' digestive system, including the cells shed from the otter's gut. Other environmental factors, such as ultra-violet radiation, enzyme activity and hydrolytic and/or oxidative damage can also act to break down cellular and genetic material in the spraint (Seutin *et al.*, 1991; Lindahl, 1993; Handt *et al.*, 1994). Therefore it is vital, if we are to be able to obtain DNA from samples, that spraint material is fresh when collected. Consequently, volunteers were asked to collect only the spraint they were confident had been laid down during the last few hours.

SURVEYORS NAME:

SAMPLE TYPE COLLECTED *Spraint/Anal Jelly/None*		**SITE NAME + CODE**	**WATERCOURSE**
TUBE NO	**DATE** **TIME**	**GRID REF**	**TRACK SIZE** (mm)
SUBSTRATE UNDER SAMPLE *Rock/Gravel/Sand/Mud/Other (describe)*		**DEGREE OF EXPOSURE OF SAMPLE** *e.g. Under bridge/Trees/Open and in Sun/Shade/dew-covered*	
WEATHER CONDITIONS ON DAY OF SURVEY		**WEATHER CONDITIONS IN PREVIOUS WEEK**	
COMMENTS *– add any information that may be relevant to the condition of the sample collected or other signs of otter activity*			

Figure 2.1 A data collection sheet showing the information recorded on site by the collection volunteers.

Close contact was maintained between the volunteers and the laboratory, with regular meetings and emails to discuss collection techniques, collection success and progress in the laboratory. This enabled the volunteers and laboratory staff to remain confident in the methods of collection being used and maintained motivation and enthusiasm in the volunteers throughout the study. As discussed later, the methods of data handling allowed easy access to, and manipulation of, data, enabling reports of the success of individual collections, as well as the success river-wide, to be easily produced. This provided collection volunteers with information, at a personal level, regarding the otters found using their collection sites.

2.2 DNA extraction

DNA extractions from tissue samples were carried out using the phenol-chloroform method of Taggart *et al.* (1992). DNA extractions from spraint material were carried out using QIAGEN mini stool extraction kits. In order to reduce the possible effects of PCR inhibitors, present in the spraint material, the spraint itself was not used in the extraction process (Hung *et al.*, 2004). Instead, the majority of IMS was poured off of the spraint and the last ~1-4ml of liquid, containing what appears to be light cellular medium, was collected in a 2ml tube. This was then centrifuged to pellet the solid material before the IMS was poured away. This process was repeated with any liquid remaining with the spraint sample before the spraint was re-immersed in 100% ethanol. The pellet of material collected in the 2ml tube was then extracted as per the QIAGEN stool extraction kit protocol.

2.3 Microsatellite genotyping

Each 10 µl PCR reaction contained 1 µl of DNA extract (increased to 2 µl for some of the spraint sample analyses), 10 mM Tris-HCl, 50 mM KCl, 1.5-2.5 mM $MgCl_2$, 0.2-0.25 mM primer (forward and reverse) and 1 U of Taq polymerase. PCR reactions were carried out using an initial stage of 94˚C for 2 minutes, followed by 35 cycles of 94˚C for 30 seconds, annealing temperature (48 - 60˚C) for 35 seconds and 72˚C for 40 seconds, before a final extension period of 10 minutes at 72˚C.

For primer optimisation, the annealing temperature, $MgCl_2$ concentration and primer concentration were modified until the best combination for producing the clearest and strongest product band were found. This ensured the best chance of successful amplification from the low quality, low quantity DNA extracted from spraint samples, ultimately increasing the chances of obtaining genotypes from the samples.

2.4 Microsatellite loci

2.4.1 Otter microsatellite primers

The majority of previous studies using otter microsatellite analysis used the suite of microsatellite primers identified by Dallas and Piertney in 1998 (Dallas *et al.*, 1999; Pertoldi *et al.*, 2001; Randi *et al.*, 2003; Hung *et al.*, 2004; Hájková *et al.*, 2006; Arrendal *et al.*, 2007). Therefore, they were the obvious choice for inclusion in the first suite of microsatellite primers to be tested. During this study a second set of Eurasian otter primers was published (Huang *et al.* 2005), providing a better choice of primers for use in this study (Table 2.1).

2.4.2 Cross-species amplification

Cross-species amplification is the use of primers, designed for use in one species, to amplify DNA fragments from another species (Slate *et al.*, 1998; Williamson *et al.*, 2002; Galan *et al.*, 2003). The method works best when species are closely related in evolutionary terms because differences between the DNA of the two species will be relatively small (Slate *et al.*, 1998; Galan *et al.*, 2003). Cross-species amplification has previously been successful across various species of mustelid (Davis & Strobeck, 1998; Kyle & Strobeck, 2001; Beheler *et al.*, 2005). In particular, Blundell *et al.* (2002) successfully used several European otter primers (Dallas & Piertney, 1998) to amplify microsatellite loci from North American river otter (*Lontra canadensis*). In this study cross-species amplification was attempted using primers designed for use in other mustelids, including wolverines (*Gulo gulo*), American mink (*Mustela vison*) and ermine (*Mustela erminea*), but by far the most successful were those originally designed for use in North American river otters (*Lontra canadensis*) (Table 2.2).

Table 2.1 Eurasian otter (*Lutra lutra*) primers tested and used in this study. Only the most informative and reliable primers were used in the spraint study.

Microsatellite locus	Repeat unit	Produce product	Used in study	Reference
Lut435	$(CA)_{29}$	Yes	Yes	Dallas & Piertney (1998)
Lut453	$(CA)_{26}$			Dallas & Piertney (1998)
Lut457 [R]	$(CA)_{26}$	Yes		Dallas & Piertney (1998)
Lut615 [R]	$(CA)_{27}$			Dallas & Piertney (1998)
Lut701 [R]	$(GATA)_{11}GAA(GATA)_2$ $GAA(GATA)_4$	Yes	Yes	Dallas & Piertney (1998)
Lut715	$(GATA)_6GAT(GATA)_7$ $GAT(GATA)_5$	Yes		Dallas & Piertney (1998)
Lut717 [R]	$(GATA)_6GAT(GATA)_7$ $GAT(GATA)_5$	Yes	Yes	Dallas & Piertney (1998)
Lut733	$(GATA)_4GAT(GATA)_{12}$	Yes	Yes	Dallas & Piertney (1998)
Lut782	$(GATA)_6GAT(GATA)_{10}$	Yes	Yes	Dallas & Piertney (1998)
Lut818	$(GATA)_{11}$	Yes		Dallas & Piertney (1998)
Lut832	$(GATA)_{11}$	Yes	Yes	Dallas & Piertney (1998)
Lut833	$(GATA)_{15}$	Yes		Dallas & Piertney (1998)
Lut902	NA			Dallas *et al.* (1999)
04OT02	$(GAAA)_{16}$	Yes		Huang *et al.* (2005)
04OT04 [R]	$(GAAA)_{16}$	Yes	Yes	Huang *et al.* (2005)
04OT05	$(GAAA)_{14}$	Yes		Huang *et al.* (2005)
04OT07	$(GAAA)_{12}GAAGG$ $(GAAA)_9$	Yes	Yes	Huang *et al.* (2005)
04OT14	$(GAAA)_{13}$	Yes	Yes	Huang *et al.* (2005)
04OT17	$(GAAA)_{13}$	Yes	Yes	Huang *et al.* (2005)
04OT19 [R]	$(GAAA)_{12}$	Yes	Yes	Huang *et al.* (2005)
04OT22	$(GAAA)_{16}$	Yes		Huang *et al.* (2005)

[R] Primer re-designed to shorten the allele fragments produced - see section 2.4.3

Table 2.2 List of the primers from related species tested and used in this study.

Microsatellite loci	Species designed for	Repeat unit	Produce product	Used in study	Reference
Gg25	Wolverine, *Gulo gulo*	$(CA)_{16}$			Walker *et al.* (2001)
Gg443	Wolverine, *Gulo gulo*	$(CA)_{14}$			Walker *et al.* (2001)
Gg452	Wolverine, *Gulo gulo*	$(CA)_{14}$			Walker *et al.* (2001)
Mvi057	American mink, *Mustela vison*		Yes		O'Connell *et al.* (1996)
Mvi087	American mink, *Mustela vison*		Yes		O'Connell *et al.* (1996)
Mvis075	American mink, *Mustela vison*	$(CA)_{12}$	Yes	Yes	Fleming *et al.* (1999)
Mer082	Stoat, *Mustela erminea*	$(CA)_{9}$			Fleming *et al.* (1999)
Mer095	Stoat, *Mustela erminea*	$(CA)_{13}$	Yes		Fleming *et al.* (1999)
Mel07	European badger, *Meles meles*	$(GT)_{21}$			Bijlsma *et al.*(2000)
RIO11	River otter, *Lontra canadensis*	$(AC)_{14}$	Yes	Yes	Beheler *et al.* (2005)
RIO13 [R]	River otter, *Lontra canadensis*	$(GT)_{20}$	Yes		Beheler *et al.* (2005)
RIO16 [R]	River otter, *Lontra canadensis*	$(GT)_{14}$			Beheler *et al.* (2005)
RIO18	River otter, *Lontra canadensis*	$(CT)_{6}$ $(CTAT)_{14}$	Yes	Yes	Beheler *et al.* (2005)

[R] Primer re-designed to shorten the allele fragments produced - see section 2.4.3

2.4.3 Re-designing primers

The degraded and fragmented state of the DNA extracted from spraint means that detection of allele sizes of around 220bp and above is often not feasible (Frantzen *et al.*, 1998). Microsatellite loci that produce larger product sizes may also be prone to higher levels of allelic dropout (Arrendal *et al.*, 2007), increasing the chances of mistyping a sample. Many of the available primers are known, from a previous study, to produce products of 200bp or greater (Dallas & Piertney, 1998) and so these primers were redesigned to create smaller product sizes (Figure 2.2).

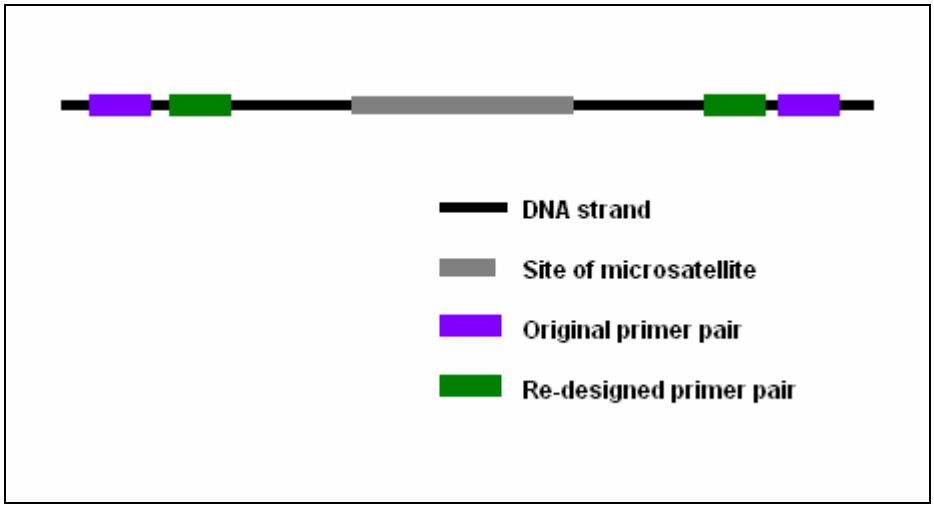


Figure 2.2 Re-designed primers sit closer to the site of the microsatellite, thus reducing the size of the fragments produced.

Table 2.3 Sequence information and levels of successful use for redesigned primers.

Microsatellite loci	Re-designed primer sequence 5'-3'	Amplified otter DNA	Used in study
Lut457b	F- ATG GCT TTA TGG CTT TCA CC R- TGT CCA CAC ATG GCC TTT C	Yes	
Lut615b	F- GTT GAA CTG GGC CTG TCT G R- TTT GCC CTT TGCT TCT GC		
Lut701b	F- TCC TTC CAT CCT TCC TTC CT *	Yes	Yes
Lut717b	F- GGG TCA AGG AGA TAC CAA GTA TG R- GGG TAA GGA CTG GAC GTT TG	Yes	Yes
Lut833b	R- GAG AGG GAG GGT GAA TGT CC *	Yes	
04OT04b	F- GGG TGG AGG TGT TAA GCA AA R- TGG GAG GCA GCA TGA TTA GT	Yes	Yes
04OT19b	F- CAC GGT GTC TGG TGT GAA AC R- CTC TGC AGC CTT TGC TTT TT	Yes	Yes
RIO13b	F- GCA CAT GGG CTT TTA TGA AGA R- TGT CCT GGG AGA GAG AGG AG	Yes	
RIO16b	F- CCC GTG GTC ACT TTA CCT GT R- TTT ATT GGG CAT GGA AGC A	Yes	

* Original primer used as complement (Dallas & Piertney, 1998)

2.4.4 Nested PCR

The technique of nested PCR is often applied in studies where DNA yields are low and copies of template DNA are scarce (Esposito *et al.*, 1998). The nested PCR method has been successfully used to genotype faecal samples; including those of brown bear (*Ursus arctos*) (Bellemain & Taberlet, 2004), and even human stools (Deuter *et al.*, 1995).

In nested PCR, an initial pre-PCR of around ten PCR cycles is carried out using an outer pair of primers (Figure 2.14). This targets the area of DNA of interest and increases the number of copies of this region. A normal length PCR process is then carried out, using the inner pair of primers and the pre-PCR mix as template DNA, (this now contains extra copies of the region of interest).. The method works by homing in on the region of interest during the pre-PCR and thus increasing the number of copies of template DNA available during the main PCR reaction.

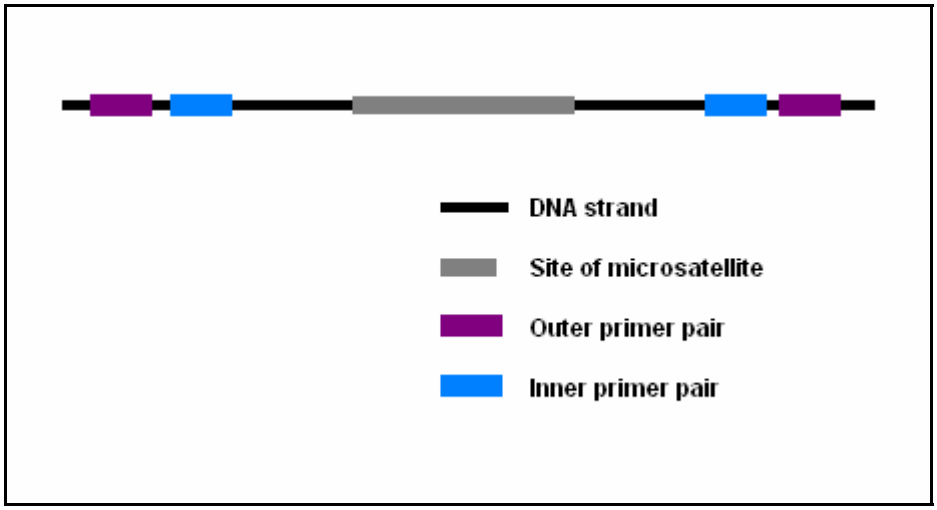


Figure 2.3 Nested PCR uses the outer primer pair in a short initial PCR cycle (pre-PCR) followed by a standard PCR cycle that uses the inner primer pair.

In this study nested PCR was attempted with three of the microsatellite loci known to produce larger PCR products. It was hoped this would improve PCR success – in particular with the failing Itchen samples. All three nested PCR reactions produced good results from DNA extracted from tissue samples. With spraint samples the technique also worked very well, resulting in strong product peaks despite the large size of the products. However, a major drawback of the nested PCR approach is that it increases the potential for contamination. Two PCR set-ups and reactions, instead of just one, and the transfer of materials from the first PCR reaction to the second greatly increases the chances of contamination occurring. This was particularly evident in the results from Lut733 where three out of the four negative controls (PCRs carried out without DNA added) produced positive results consistent with a contamination event.

Although the results from the nested PCRs were good the risk of contamination was considered too high and only the results from Lut832, the majority of which were obtained using non-nested PCR, were used in the final spraint genotype analysis. Lut733 and Lut782 were used, in non-nested PCR, for River Itchen spraint samples, but were dropped for the River Camel spraint sample analysis in favour of more productive loci.

2.4.5 The final primer set

The final suite of primers used for genotyping the River Camel spraint samples and the tissue samples includes nine microsatellite primers designed for use in otters; four have been re-designed to produce smaller product sizes and two were originally designed for use in North American river otters (*Lontra canadensis*). The River Itchen spraint samples, which were genotyped towards the beginning of the study, before all of the primers had been fully optimised, were genotyped using the primers Lut435, Lut717b, Lut832, 04OT14 and 04OT17, plus three primers which were only used for the River Itchen samples; Lut733, Lut782 and Mvis075.

Table 2.4 List of microsatellite loci analysed as part of the genotyping of spraint and tissue samples.

Microsatellite loci	Number of alleles	Product size range	Annealing temperature	Reference
Lut435	4	125-139	60˚C	Dallas & Piertney (1998)
Lut701b *	4	147-159	58˚C	Dallas & Piertney (1998)
Lut717b *	4	98-118	60˚C	Dallas & Piertney (1998)
Lut733	4	157-179	58˚C	Dallas & Piertney (1998)
Lut782	3	164-214	58˚C	Dallas & Piertney (1998)
Lut832	5	181-197	60˚C	Dallas & Piertney (1998)
04OT04b *	4	164-196	53˚C	Huang *et al.* (2005)
04OT07	6	206-226	53˚C	Huang *et al.* (2005)
04OT14	4	118-142	60˚C	Huang *et al.* (2005)
04OT17	3	143-159	60˚C	Huang *et al.* (2005)
04OT19b *	3	180-188	50˚C	Huang *et al.* (2005)
RIO11	3	150-156	58˚C	Beheler *et al.* (2005)
RIO18	2	181-185	58˚C	Beheler *et al.* (2005)
Mvis075	2	125-128	58˚C	Fleming *et al.* (1999)

* Primers redesigned to produce a smaller product size

Previous studies of otter populations using genotypes obtained from spraint material have used between six (Hájková *et al.*, 2006) and nine loci (Dallas *et al.*, 2003; Lampa *et al.*, 2008). The use of genotypes, obtained from tissue samples, to calculate measures of Probability of Identity (PI) has shown that nine loci (plus the SRY gene) are sufficient to accurately genotype spraint samples, with the likelihood of two samples from different but related individuals expressing the same genotype (PI_{sibs}) being between 2.33×10^{-3} (Arrendal *et al.*, 2007) and $5.6\text{-}6.8 \times 10^{-3}$ (Dallas *et al.*, 2003). These figures suggest that the eleven loci analysed here are excessive. However, the accuracy of identification of individuals via genotyping is also reliant on the levels of polymorphism expressed in the loci used. As many of the loci used in this study express relatively low levels of polymorphism we opted to use more loci. In

addition, although the likelihood of two individuals expressing the same genotype at nine microsatellite loci is apparently low, the phenomenon was observed by Coxon *et al.* (1999). They observed identical genotypes in both the carcass of an otter and in spraint samples collected both before and after the otter's death.

In summary, the findings of our work agree with previous studies and indicate that it is advisable to use as many loci as is feasible for a particular study. Such a strategy increases the chances of being able to distinguish between individuals, particularly in situations where it is likely that closely related individuals have been sampled. Given the low levels of polymorphism observed for the loci used in this study, our use of eleven microsatellite loci is not excessive.

The suite of primer pairs used for the analysis of spraint are all otter specific (or specific to a related otter species not present in this country) and are unlikely to amplify bands of a similar size from prey species. The most likely species the primers might amplify are the relatively closely related American mink (*Mustela vison*) and polecat (*Mustela putorius*) (Slate *et al.*, 1998; Kyle & Strobeck, 2001; Galan *et al.*, 2003). The scat from these species is sometimes mistaken for otter spraint (Hansen & Jacobsen, 1999). However, any mistaken identity should be easily observable in the results, as the allele sizes from these species are unlikely to match the allele sizes expected from otter samples (Davis & Strobeck, 1998; Beheler *et al.*, 2005).

2.5 Genotyping spraint samples

For spraint genotyping the number of PCR cycles was increased to 45 to increase the number of copies present at the end of the process. This increases the sensitivity of the technique, but also increases the risk of amplifying contaminant fragments of DNA.

A problem often encountered during microsatellite analysis of low DNA yields, such as those obtained from spraint samples, is a phenomenon known as allelic dropout. This occurs when there is so little template DNA present that only one of the alleles is amplified during the initial stages of the PCR and so only this allele is observed when analysing the PCR results. The effect of allelic dropout is to make it appear that the specimen is homozygous (has two alleles of the same size) at that locus. In fact it may be heterozygous (have two alleles of differing sizes), but only one allele has been amplified in the PCR.

In order to identify, and counteract, instances of both contamination and allelic dropout a set of simple procedures were followed. The method, based on the multi-tube approach of Taberlet *et al.* (1996), was designed to be easy to use and follow, with saving of time and money in mind, while still obtaining the best possible results from a difficult and limiting resource. Figure 2.4 shows the steps followed, after DNA extraction, to successfully genotype samples.

A full genotype was deemed to have been acquired when, for each locus, either:

- One allele was observed three or more times, with no other alleles observed, or
- Two alleles were observed three or more times each.

If a third allele was observed then an allele that was seen only once was considered to be due to contamination. If a second allele was seen only once, when the first allele was observed five, or more, times, this second allele was considered to be due to contamination.

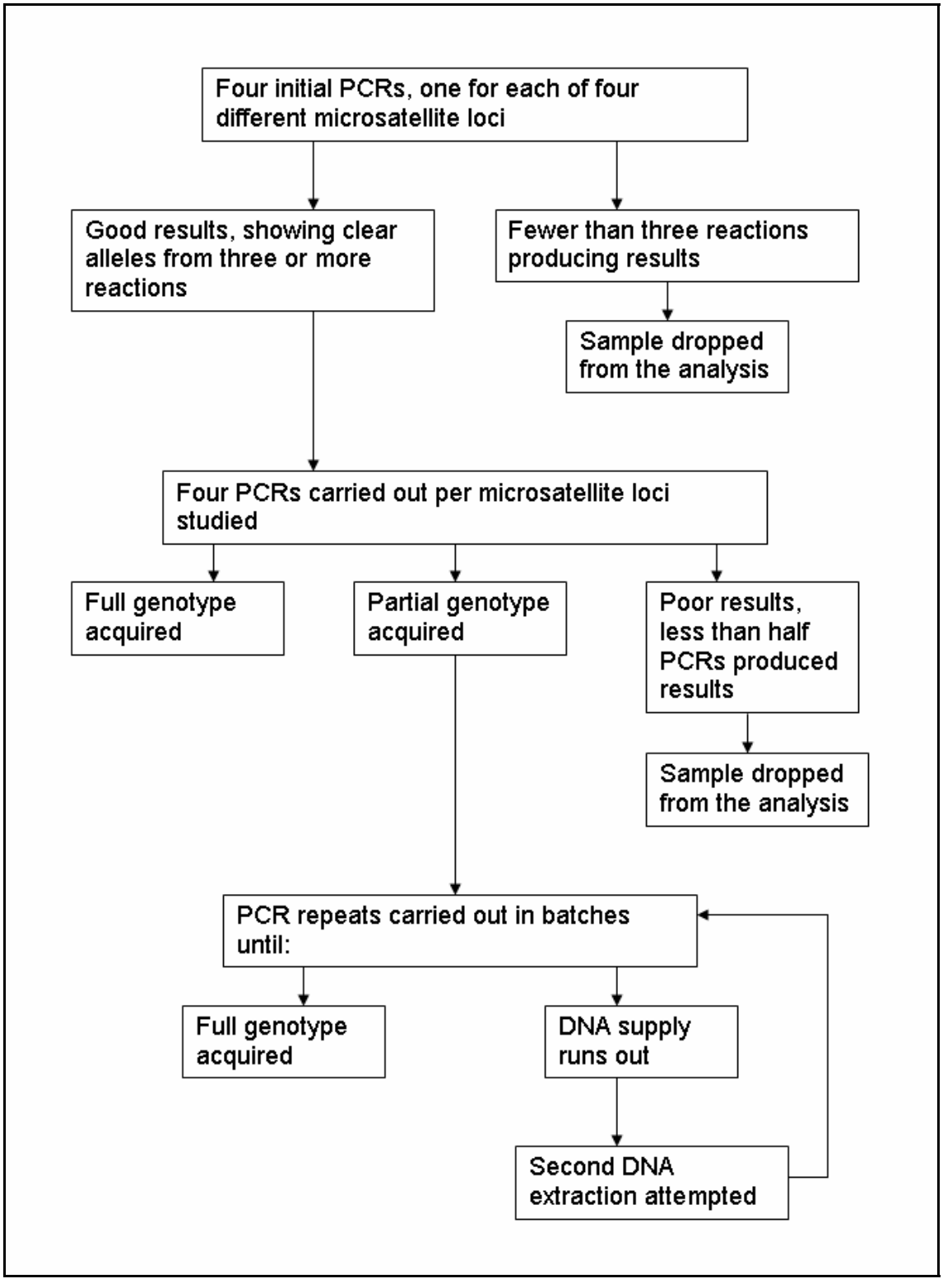


Figure 2.4 Steps followed during the processing of spraint sample DNA extractions.

2.6 Collation of spraint sample data

Large volumes of data were collected per spraint sample, this included field collection data, multiple PCR and final genotype data, and an effective system for data storage and processing was required. An Access database was designed that was capable of holding, and linking, all the data relating to each sample. The table layouts can be seen in Appendix 1. The database also allows for querying and sorting of samples according to particular criteria, without the loss of data relating to the samples. In particular this was useful for collating multiple PCR results into one genotype.

2.7 Obtaining mitochondrial haplotypes

Primers L-Pro and 363rev (Ketmaier & Bernardini 2005) were used to sequence >300bp from the 5´ end of the mitochondrial control region. This marker has been used in a number of previous studies of Eurasian otter populations (Mucci *et al.* 1999; Cassens *et al.* 2000; Ferrando *et al*. 2004; Pérez-Haro *et al* 2005) and has been shown to be highly informative in discriminating otter haplotypes. Polymerase chain reaction (PCR) amplifications were carried out in a 25µl reaction volume containing 10mM Tris-HCl (pH 8.3), 50mM KCl, 1.5mM $MgCl_2$, 0.2mM of each dNTP, 0.75µM (0.75pmol.μl^{-1}) of each primer, 1U of REDTaq DNA polymerase (Sigma-Aldrich) and 2µl of DNA extract. The PCR program was a denaturing stage of 94ºC for 2min, 30 amplification cycles of 94ºC for 15secs, 50ºC for 15secs and 72ºC for 45secs, followed by a final extension period of 5min at 72ºC.

Six PCR reactions were performed on template DNA from each sample to eliminate possible sequencing artefacts; products were then separated on 1% agarose gels. Reactions produced single bands of approximately 365bp. These were excised and extracted using a QIAGEN gel extraction kit. The resulting DNA products were then pooled and sequenced in both directions (Lark Technologies) using the L-Pro and 363rev primers. The forward and reverse sequences for each sample were aligned using the AutoAssembler v.2.0 program (Applied Biosystems) to obtain a sequence of >300bp. The sequences were then aligned with each other and with previously defined haplotypes (Lut1-5 from Cassens *et al* 2000, GenBank accession no. AJ006174-78; Lut 6 from Pérez-Haro *et al* 2005). Alignments were performed using the program ClustalX (Thomson *et al* 1997). All alignments were checked by eye to eliminate alignment artefacts.

3 Analytical chemistry methods

3.1 Liver tissue collection

The Environment Agency, aided by local organisations, such as the Wildlife Trusts, and members of the general public, have been collecting otter carcasses from the south west of England since 1988 (Simpson, 1998) and from across England and Wales since 1992 (Chadwick, 2007). Carcasses have been collected primarily to collect liver tissue for the analysis of levels of PCBs and organochlorines (Simpson, 1998; Simpson, 2000; Chadwick, 2007). In addition, *post mortems* also reveal information about the general health of the otters collected, with age, sex, general body condition and cause of mortality all noted where possible (Philcox *et al.*,1999; Madsen *et al.* 1999; Hauer *et al.* 2002; Chadwick, 2007). Over the years a variety of pathological and histological analyses have been carried out on a range of tissue samples (Simpson, 1998; Simpson, 2007).

Recent *post mortem* examinations – those concerning samples used in this study – were carried out in one of two locations. A degree of overlap occurred between sample collection sites and the location of their *post mortem*. However, the majority of samples from the south west of England were examined at the Wildlife Veterinary Investigation Centre (WildlifeVIC) in Cornwall (Simpson, 2007) and the majority of samples from the north, east and midlands of England, as well as samples from Wales, were processed by the Cardiff University Otter Project (CUOP) (Chadwick, 2007).

As with the kidney tissue samples collected for genetic analysis, liver tissue samples for chemical analysis were collected as part of *post mortem* examinations. Liver tissue samples were originally collected for analysis of PCB and selected organochlorines in Environment Agency laboratories. In many cases duplicate samples were taken and the second (and sometimes third) samples were stored frozen by the *post mortem* teams. It was these duplicate samples that became available for PBDE analysis . Samples were stored wrapped in foil and placed in individual plastic bags before freezing to minimise the risk of contamination, either from the environment, from other samples or, in the case of the foil wrapping, from the plastic bags themselves.

3.2 PBDE extraction and sample clean-up

To reduce contamination all glassware used for samples was cleaned using a cleaning solution (Decon) and warm water before being rinsed in millicon purified water and oven dried. Glassware was then placed in a furnace at 400ºC overnight. All glassware was rinsed in acetone, then hexane, then DCM before use. Glassware which could not be cleaned in this manner, such as glass pipettes and GCMS vials, was baked overnight at 400ºC to remove as much contamination as possible.

As deca-BDE is light sensitive it was important to keep UV radiation to a minimum throughout the extraction and clean-up process. Amber glassware was used whenever

possible, combined with methods of covering and/or storing the samples that kept exposure to a minimum.

Samples were extracted in batches of six, each batch included a blank to control for contamination levels. Care was taken to ensure that liver tissue taken for analysis did not include material from the outer 5mm of the main sample. These areas are the most likely to be contaminated and the most likely to have defrosted during any sample transfer operations. Samples of approximately 5g of liver (1g of seal blubber) were weighed on to tin foil before being chopped finely and put in a blender with a measure of anhydrous sodium sulphate. The sodium sulphate must previously have been baked at 400ºC overnight in order to make it anhydrous and capable of absorbing the water from the liver sample. The mixture of liver and sodium sulphate was blended until roughly homogenised and then stored in a clean and covered glass beaker ready for extraction. In between samples any equipment that came into contact with the sample, including the metal bell of the blender, was washed in cleaning solution and warm water, then rinsed in acetone, hexane and then DCM. Blank samples were prepared by adding a small amount of anhydrous sodium sulphate to the cleaned sampling equipment before a larger amount of sodium sulphate was added and blended briefly before storage and extraction.

Soxhlet thimbles were pre-extracted in the soxhlets for 2-4 hours before being used for the extraction of samples. Large thimbles were required to accommodate the sample material - including the relatively large amount of sodium sulphate. Samples were spiked with 25µl of internal standards (20 ng.µl^{-1} of $^{13}C_{12}$-PCB-153, -138, -180 and -209 and 20 ng.µl^{-1} of $^{13}C_{12}$-BDE-209 and BDE-49 and -51) before being soxhlet extracted overnight using DCM as a solvent. Extractions were carried out in a room with UV filters on the windows and foil was wrapped around the flasks and soxhlet bodies to reduce the exposure of sample to light.

Approximately 10% of the extract was removed for sample lipid determination. The exact percentage of sample removed was determined by weight; thus, final concentrations could be adjusted accordingly. The sample lipid content was calculated using the weight of the extract once dried.

The remaining sample (not used for lipid determination) was reduced and transferred using hexane washes to a clean test tube. The sample was then cleaned-up by mixing it thoroughly with an equal volume of 15% fuming sulphuric acid, before centrifugation to separate the two phases. The lower sulphuric acid phase was then removed and the process was repeated until the acid phase was clear (approximately 7 cycles). Approximately 1ml of purified water was then added and mixed with the sample before centrifugation and removal of the water layer. A small amount of anhydrous sodium sulphate was then used to absorb any water remaining in the sample before it was transferred, with hexane washes, to an amber vial.

The sample, held in an amber vial in hexane, was reduced down to a few drops before being eluted through a GPC column (35mm d, 70cm l, filled with 70g Biobeads) with 1:1 DCM:hexane at approximately 5ml/min. After rejection of the first 16ml fraction, the next 30ml fraction was collected. This was then reduced down and transferred to pure hexane before transfer to a GCMS vial containing 25µl of dodecane containing the internal standards, BDE-69, BDE-181, PCB-30, $^{13}C_{12}$PCB-141 and $^{13}C_{12}$PCB-208.

3.3 GCMS analysis

Analysis of the samples for PBDE congener content was carried out using gas chromatography/mass spectrometry (GCMS). Analysis of the lower congeners (BDE-17, -28, -32, -35, -37, -47, -66, -71, -75, -77, -85, -99, -100, -119, -128, -138, -153, -154, -166, -183, -190, -196 and-197) was carried out using a Fisons MD800 GC-MS used in SIM mode. The GC column was a standard DB-5 column, 30m long with a 0.18mm id. The mass spectrometer was used in ECNI (electron capture negative ion) mode with ammonia as the reagent gas. 1µl of analyte solution was injected, in splitless mode, into a split/splitless injector that was maintained at 300ºC. The temperature program, used under constant pressure, was: hold at 100ºC for 2 min, 4ºC/min to 300ºC and 10ºC/min up to 320ºC. The GCMS interface temperature was 270ºC and the ion source temperature 300ºC. Concentrations of nona and deca-BDEs (BDE-206, -207, -208 and -209) were determined using a HP6890 GC attached to a VG Autospec Ultima used in EI+ mode, with a resolution of at least 10,000. Separation was achieved on a 15m long 0.18mm id, DB5 MS column. PCBs and OCs were analysed on a Thermo Trace GC-MS in SIM mode using an EI+ source. Separation was achieved on a 50m 0.25mm id CP-Sil 8 column.

4 Genetic analysis of spraint

4.1 River Itchen results

4.1.1 Number of otters observed

A total of 104 spraint samples were collected from across the River Itchen catchment. DNA extraction was attempted from 59 of these, but only four produced sufficiently high DNA concentrations to be genotyped. Three other sample extracts showed signs of containing DNA, but the yield was low and the samples only produced partial genotypes. If only those samples that gave a full genotype are considered this is a success rate of 6.8%. Including the partial genotypes increases the success rate to 11.9%. However, this is still far below what would be expected and suggests that there is an underlying problem with the samples collected from the River Itchen catchment.

The four samples that were successfully genotyped each produced a different genotype (Appendix 2.1), so the four samples represent four different otters. As all four genotyped samples were collected in May 2006 it seems that at least four otters were using the catchment during this short time period. This is a minimum number and the actual number that the river is supporting at any one time is likely to be much larger.

4.1.2 Possible reasons for lack of success

It is not clear what caused the low success rates with the River Itchen samples. Several of the collection volunteers had experience collecting samples for a previous successful spraint genotyping study (Coxon *et al.*, 1999; Coxon *et al.*,2003; Chanin, 2004) and so their collection techniques and handling of samples in the field would not be expected to be at fault. As the samples were not treated any differently to the River Camel samples, it seems unlikely that the laboratory treatment was at fault.

One major difference between the River Itchen and River Camel samples was the medium used to store the samples after collection. During the first few months of collection the River Itchen samples were stored in industrial ethanol. Impurities in industrial ethanol were suspected to be the cause of the low success rates and the collection medium was changed to biological grade ethanol. However, this did not improve success rates. The collection tubes and storage medium were then changed to match exactly those used for the successful River Camel collections, but this also failed to improve success rates. The problems encountered with the River Itchen samples do not seem to be related to the collection medium used. Indeed, the most successful samples were collected during the first few months of collections, while the industrial ethanol was still in use.

Nevertheless, to optimise conditions for spraint DNA preservation, it is recommended that collection tubes are prepared in the laboratory with biological grade ethanol (95%) to control for possible collection medium effects.

The lack of improvement in success rates with the use of collection and storage techniques that matched those used on the River Camel and the lack of any obvious field or laboratory based problem with the handling of the samples, suggests that there is some other, unidentified, reason for the low success rates obtained from the River Itchen samples.

4.2 River Camel results

4.2.1 Number of otters observed

DNA extractions were made from 227 of the 499 spraint samples collected from the River Camel catchment. Of these 41 produced a genotype - representing an 18.1% success rate. Three other samples produced partial genotypes that could be assigned to individual otters, but with a much lower level of confidence (see Appendix 2.2).

From the genotyped samples 16 different otters, each represented by a unique genotype variation, could be identified. Nine different genotypes were found in the first collection season and twelve in the second. This means that a minimum of nine otters used the River Camel at some point during the period from October 2005 to May 2006, and a minimum of twelve otters used the catchment at some point during the period of October 2006 to June 2007. From this we can conclude that the River Camel is capable of providing, at least in part, for the needs of at least twelve different otters during a nine month, over-winter period.

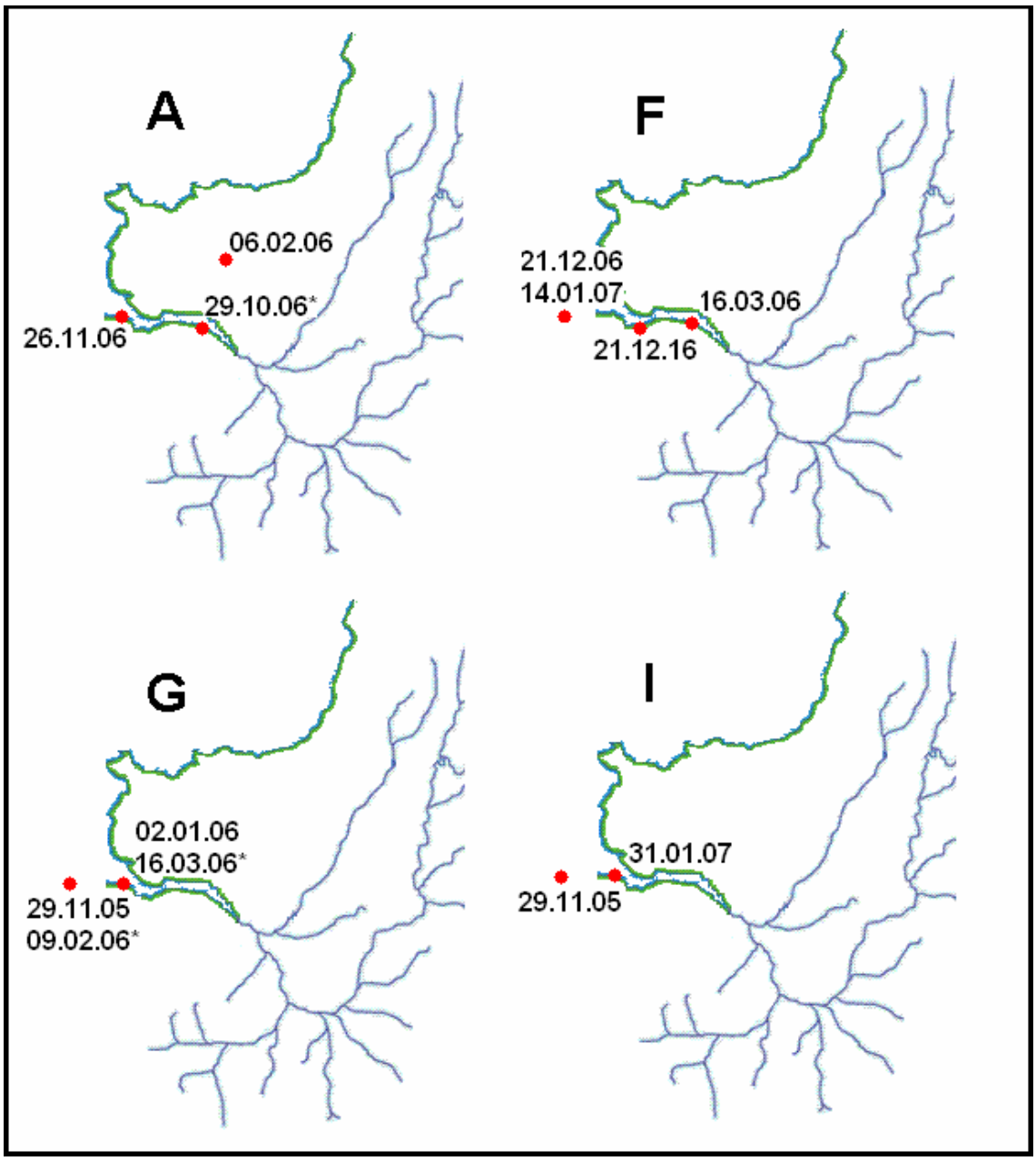
The sampling effort by collection volunteers was not uniform across all areas of the catchment or across time. Some areas of the catchment were covered extremely well, in particular the estuary and lower reaches of the catchment. In these areas volunteers were able and willing to spend time sampling regularly and thoroughly. In other areas volunteers were not able to spend as much time collecting and/or samples were much harder to find and collect. Consequently, in these areas samples were often collected from point sources over a relatively short period of time, making the coverage less intense and less uniform. This imbalance in sampling is likely to have resulted in some otters being unrecorded in areas of lower sampling effort. In particular, otter numbers are likely to have been underestimated in the upper and middle reaches of the River Camel and along one of the river's major tributaries, the River Allen.

4.2.2 Dates and locations

Using the information on dates and locations of sample collections it was possible to map the location and movement of individual otters and several distinct areas of otter activity were identified. One group of otters used the estuary (Figure 4.1), a second an

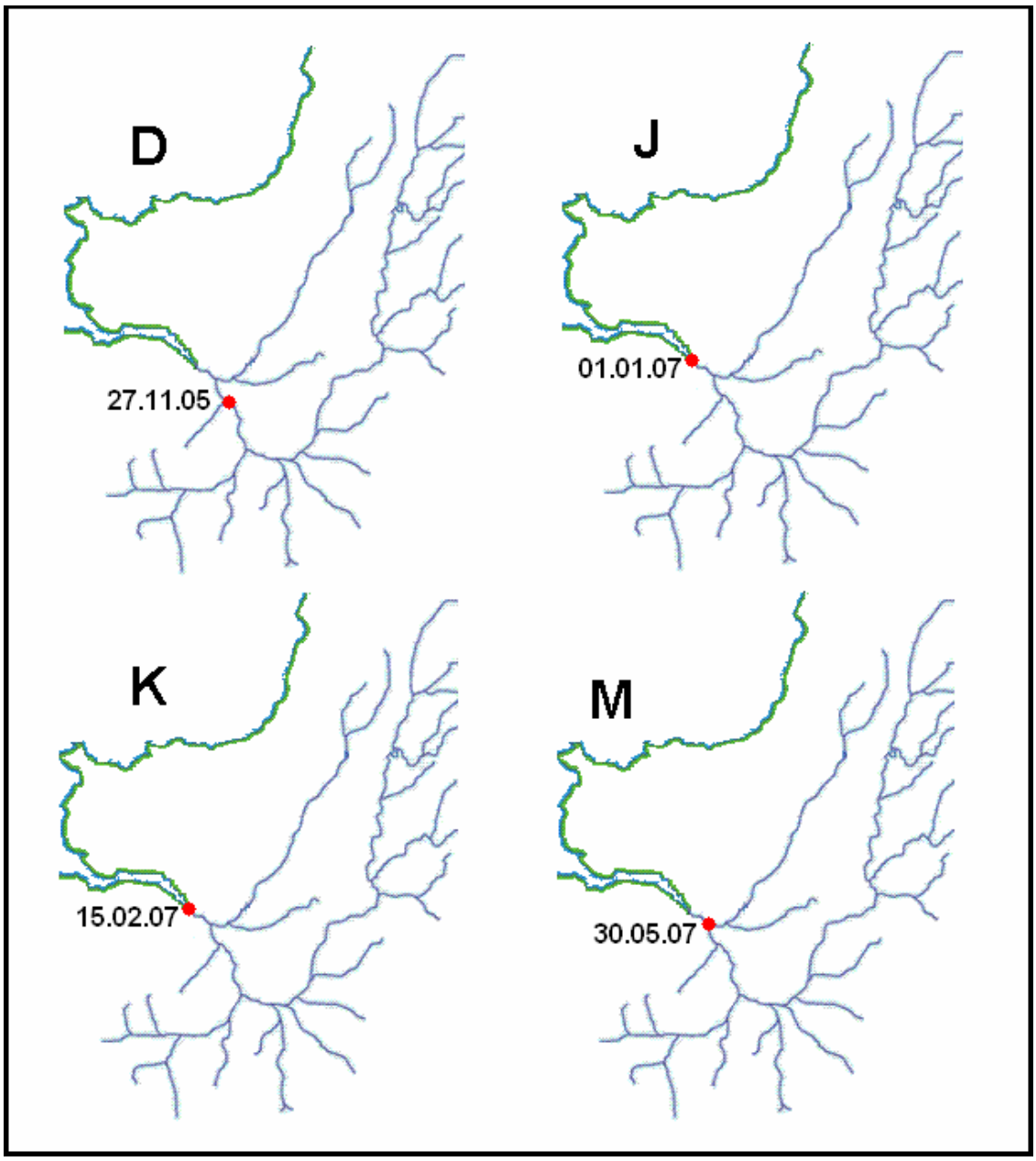
area just above the estuary (Figure 4.2), a third the lower to mid-reaches (Figure 4.3) and a fourth group the upper reaches (Figure 4.4). None of the otters were found using more than one of these areas, indicating that they were very site specific in their use of habitat/home ranges (Erlinge, 1967; Ruiz-Olmo *et al.*, 2005).

Of note is sample CAM145, collected from the Gam Bridge site in the upper reaches of the Camel catchment on the 7 March 2006. This was assigned to otter A and may represent movement of this individual across the catchment. However, sample CAM145 was only genotyped at four microsatellite loci, so the confidence in the assignment of otter identity is significantly lower and should not be relied upon.



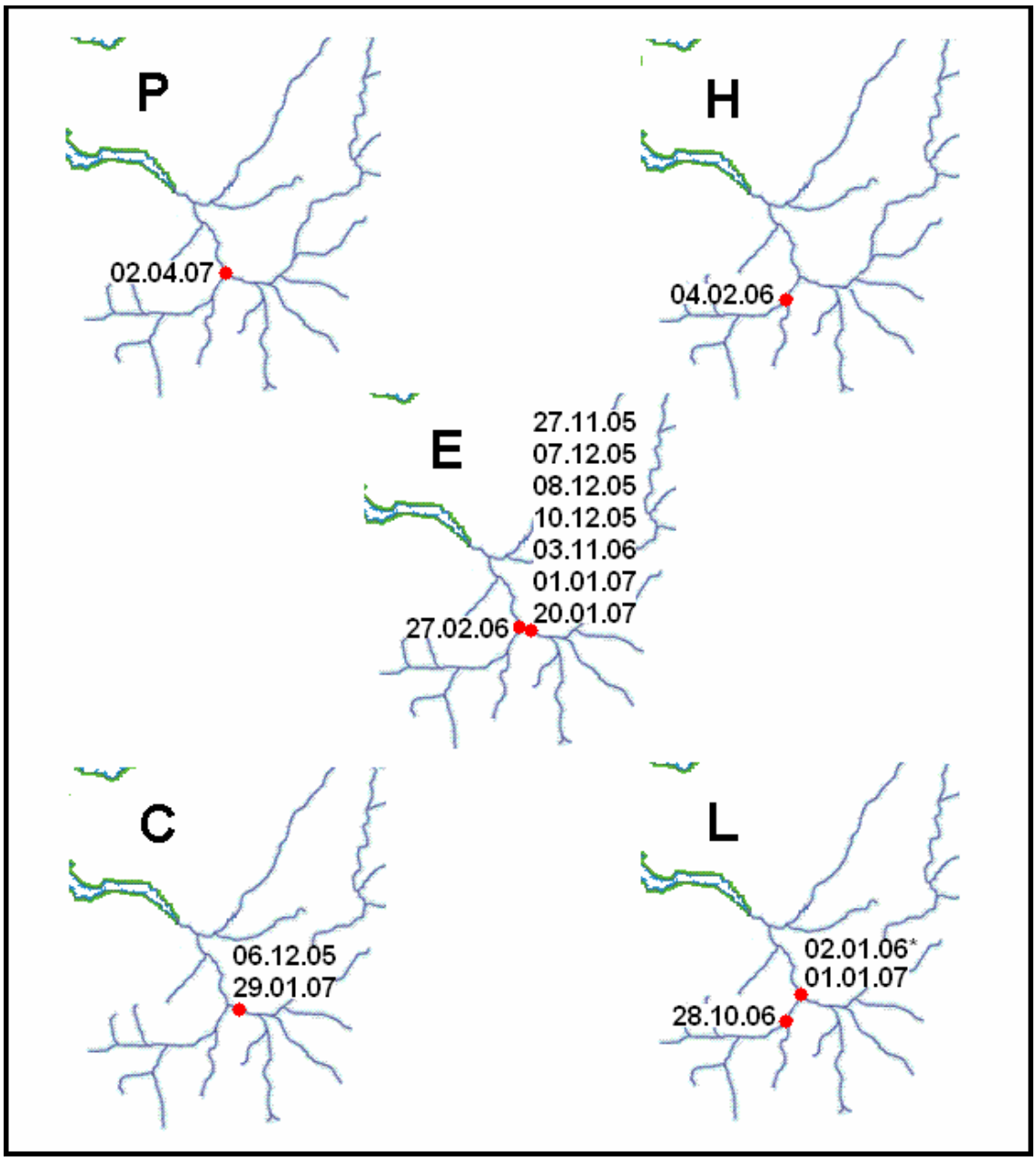
Otter	2005-2006								2006-2007								
	O	N	D	J	F	M	A	M	O	N	D	J	F	M	A	M	J
A																	
F																	
G																	
I																	

Figure 4.1 Dates and locations for the collection of spraint samples identified via microsatellite genotyping as the individuals labelled A, F, G and I, all of which were found to be using the estuary of the River Camel. Stars denote samples for which there are questions over the assignment. Underneath is a map of the months in which these otters were found. Dark squares denote positives, striped squares represent genotypes that may belong to a separate individual and hatched squares represent poorly genotyped samples that fit only this individual for the few loci that have been typed.



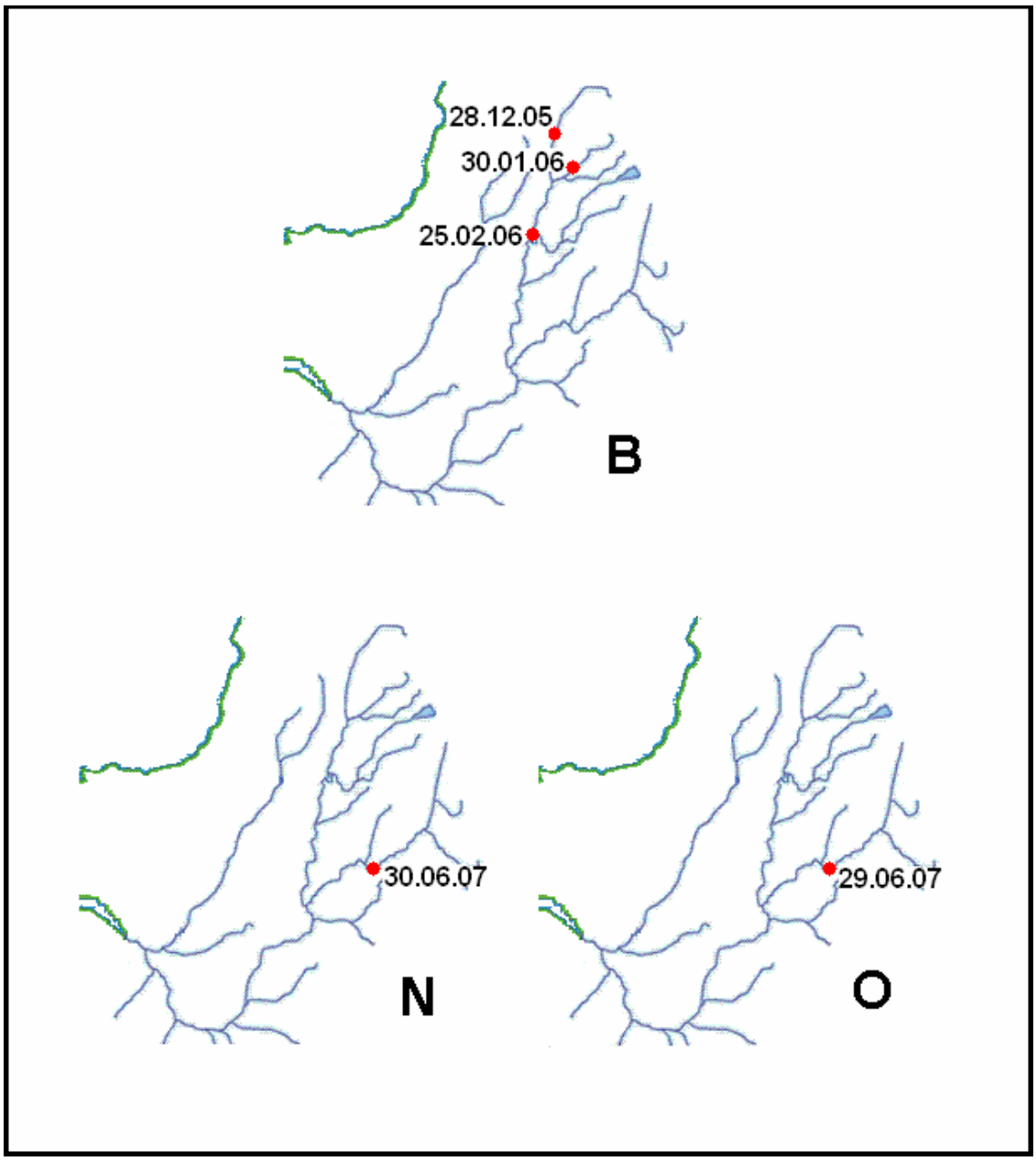
Otter	2005-2006								2006-2007								
	O	N	D	J	F	M	A	M	O	N	D	J	F	M	A	M	J
D		■															
J												■					
K													■				
M																■	

Figure 4.2 Dates and locations for the collection of spraint samples identified via microsatellite genotyping as the individuals labelled D, J, K and M, all of which were found to be using the lower section of the River Camel. Underneath is a map of the months in which these otters were found, Dark squares denote positives.



Otter	2005-2006								2006-2007								
	O	N	D	J	F	M	A	M	O	N	D	J	F	M	A	M	J
P															■		
C			■									■					
E		■	■		■					■		■					
H					■												
L				▨					■			■					

Figure 4.3 Dates and locations for the collection of spraint samples identified via microsatellite genotyping as the individuals labelled P, H, E, C and L, all of which were found to be using the lower to mid section of the River Camel. Underneath is a map of the months in which these otters were found. Dark squares denote positives and hatched squares represent poorly genotyped samples that fit only this individual for the few loci that have been typed.



Otter	2005-2006								2006-2007								
	O	N	D	J	F	M	A	M	O	N	D	J	F	M	A	M	J
B			■	■	■												
N																	■
O																	■

Figure 4.4 Dates and locations for the collection of spraint samples identified via microsatellite genotyping as the individuals labelled B, N and O, all of which were found to be using the upper reaches of the River Camel. Underneath is a map of the months in which these otters were found. Dark squares denote positives.

4.2.3 Inferred relationships

Measures of pairwise relatedness can be used to gain information about how closely related two individuals are to each other. The measure compares the variation and similarities in allele frequencies of the two individuals in question with that of the population. This provides a measure of how much of the similarity between the genotypes is due to chance and how much is likely to be due to a true genetic relationship between the two individuals. The results of pairwise relatedness analysis for the River Camel spraint genotypes are presented in Table 4.1. Relatedness values of 0.5 or above are highlighted as these represent pairs of individuals that are likely to be related as parent-offspring or as full siblings.

Using the values of pairwise relatedness it is possible to draw up several family groups, in which several individuals are related to each other to differing degrees (Figures 4.5, 4.7 and 4.9). Parentage analysis log likelihood (LOD) scores, obtained using the genetics computer program Cervus (Marshall *et al.*, 1998), provide a likelihood of the relationship being that of a parent and offspring. Although the sample numbers used are probably too low for this method to provide an accurate account of relationships, it does give an idea of relationship patterns, with pairs of individuals with high LOD scores being more likely to be parent and offspring than those with lower scores. However, the method is based on allele sharing and so high scores may be obtained by chance.

Care must be taken when interpreting scores of relatedness and parentage. The values given represent the likelihood of there being a relationship, given the allele frequencies observed, and should not be taken as direct evidence of there being a true relationship.

Table 4.1 Relatedness values for the pairs of otters genotyped from the River Camel catchment. Pairwise relatedness values above 0.50 are highlighted, indicating the pairs of individuals that show a high probability of being related.

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
B	-0.476	*													
C	-0.588	0.383	*												
D	-0.354	0.121	-0.028	*											
E	0.262	-0.496	-0.120	-0.147	*										
F	**0.510**	-0.510	-0.926	-0.238	-0.013	*									
G	-0.545	-0.094	0.134	-0.073	0.143	-0.389	*								
H	-0.292	0.066	-0.068	0.340	-0.122	-0.239	0.238	*							
I	-0.205	-0.370	-0.462	-0.256	-0.349	**0.589**	-0.492	-0.246	*						
J	0.133	-0.150	0.097	-0.014	0.124	0.248	-0.328	-0.050	0.462	*					
K	0.362	-0.271	-0.579	-0.192	-0.593	**0.725**	-0.642	-0.194	**0.614**	0.355	*				
L	-0.439	0.293	0.069	0.433	-0.075	-0.197	0.232	**0.825**	-0.092	0.285	-0.271	*			
M	0.453	-0.201	-0.764	0.221	-0.177	**0.674**	-0.629	-0.059	0.330	0.201	**0.637**	-0.132	*		
N	0.056	0.149	0.179	0.434	0.203	-0.375	-0.083	-0.082	-0.679	-0.142	-0.518	0.100	-0.042	*	
O	-0.310	**0.573**	0.443	-0.521	-0.492	-0.179	-0.328	-0.227	0.272	0.414	0.286	0.112	-0.373	-0.292	*
P	-0.360	-0.298	0.086	-0.080	0.317	-0.044	0.398	0.381	0.238	0.469	-0.250	0.455	-0.296	-0.429	-0.017

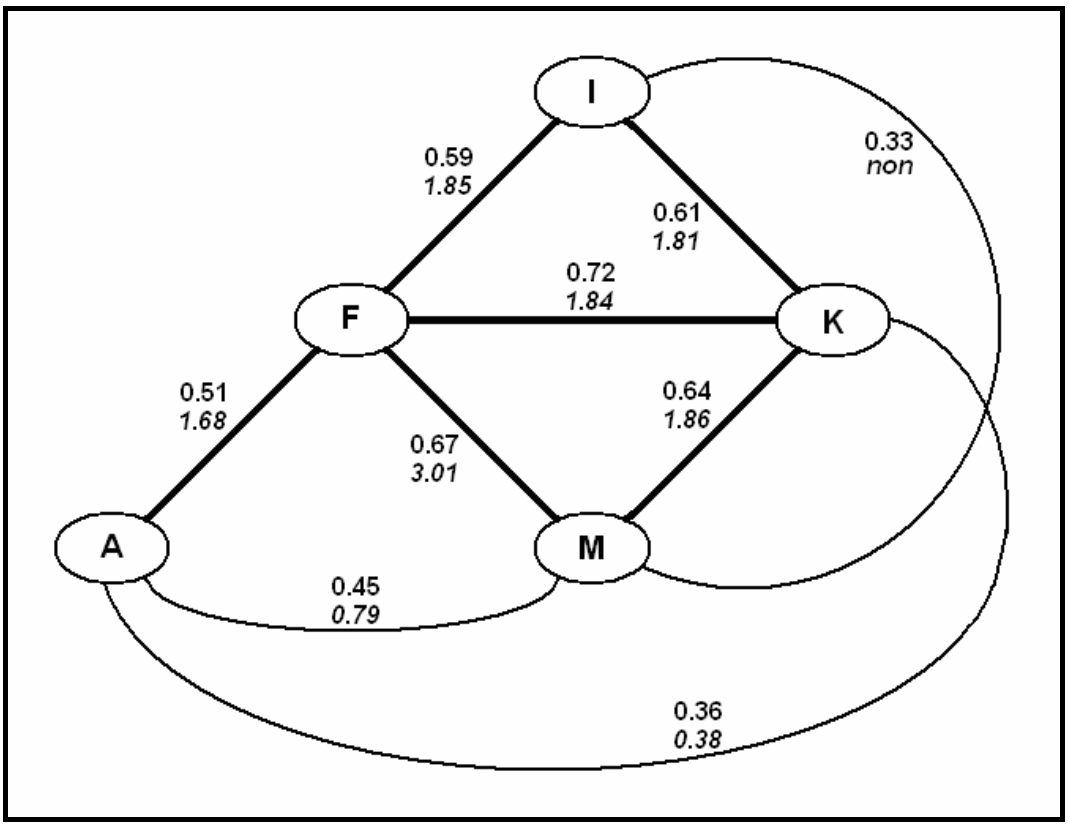
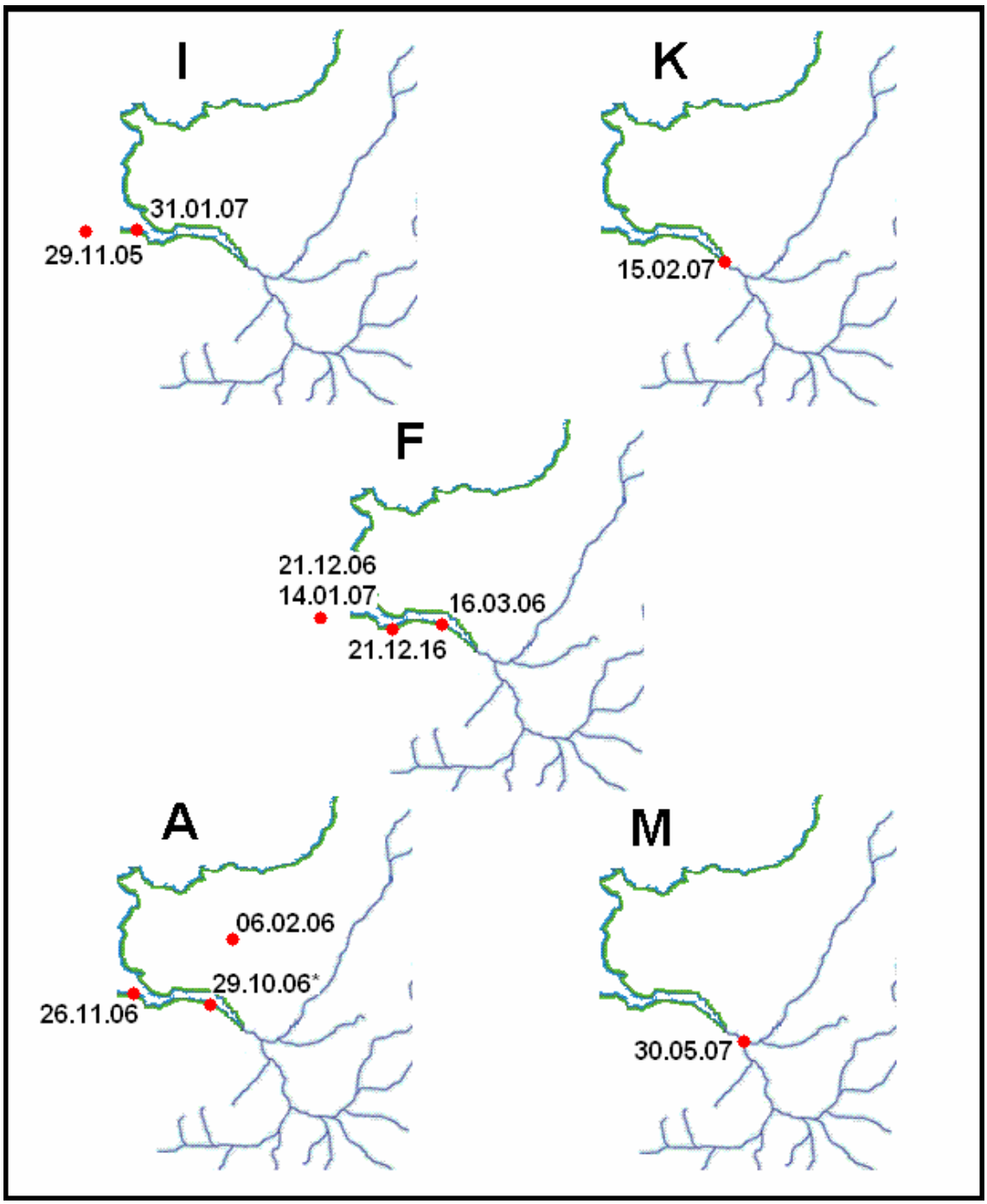


Figure 4.5 Presumed relationships between otters marked A, F, I, K and M, showing pairwise relatedness values, with LOD (log likelihood) scores for parentage analysis shown in italics underneath. For both measurements, the higher the value, the more likely it is that there is a genuine genetic relationship between the two linked individuals.

The results of pairwise relatedness and parentage analysis suggest that otters A, F, I, K and M are likely to belong to the same family unit. Otters F, I, K and M all share high relatedness and parentage scores with each other, with the exception of otters I and M. These show an ambiguous value of relatedness (r = 0.33) and a non-existent parentage analysis score, suggesting these two otters are related in a non-parent-offspring bond. Perhaps, given the relatively low values of relatedness (when compared to other pairs in the group) theirs is a secondary bond, such as half siblings or cousins. Otter A shows a high level of relatedness to otter F and, to a lesser degree, to otters M and K, suggesting that otter A may be related to the rest of the family group through its close relationship to otter F.

Without information regarding the age, gender and reproductive status of the otters it is difficult to be certain of family relationships. However, the values of genetic relatedness suggest that this group of otters does form a close family group. Information about the locations in which spraint from these otters were found (Figure 4.6) indicates that all the otters identified in this supposed family group inhabit the lower section of the River Camel, including the estuary. This location data adds confidence to the proposed family relationships. Aside from migratory events - for example when young otters leave their mother to find their own territories - families (particularly mothers and cubs) would be expected to be found in the same location. The non-random nature of the location data supports the family relationships, derived from the genotype data, and makes it more likely these represent true genetic ties.



Otter	2005-2006								2006-2007								
	O	N	D	J	F	M	A	M	O	N	D	J	F	M	A	M	J
I		■										■					
K													■				
F						■					■	■					
A					■				▨	■							
M																■	

Figure 4.5 Dates and locations for five otters that show significant ties of pairwise relatedness. Underneath is a chart showing the months in which these otters were sampled, as shown by the dark squares. The striped square represents a genotype which may belong to a separate individual.

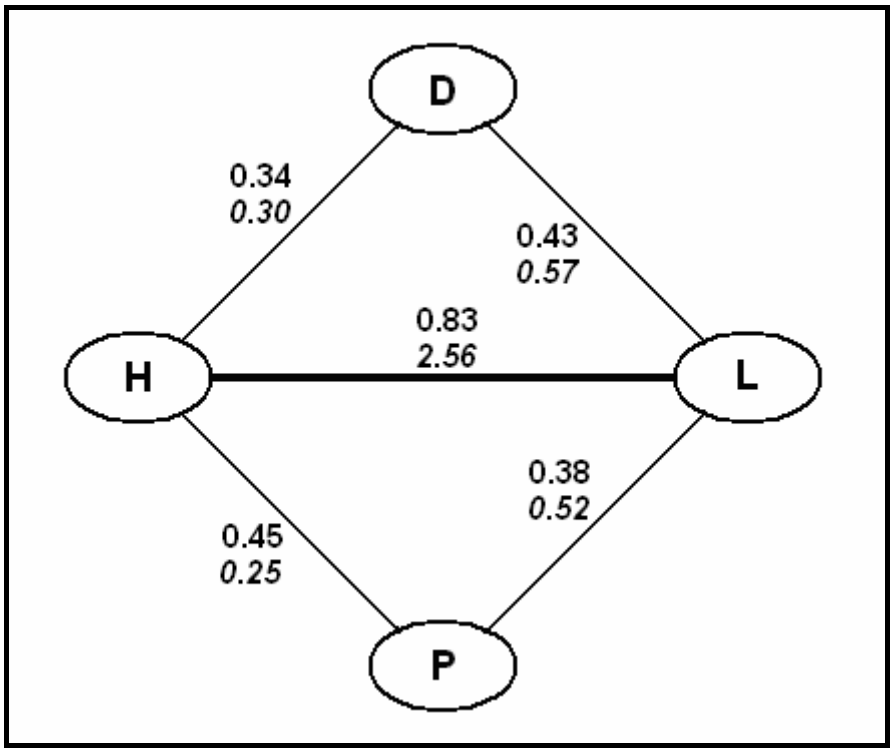
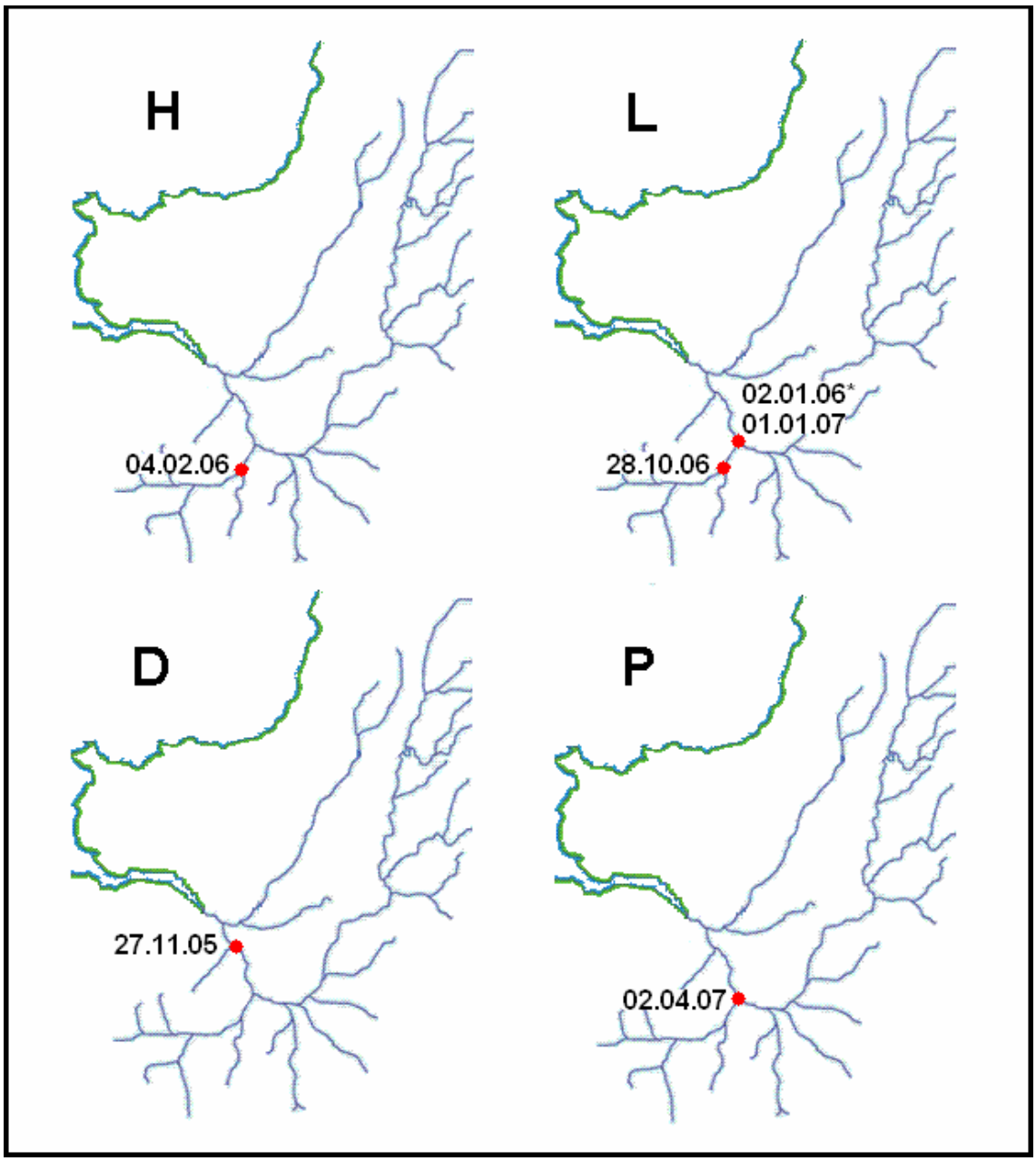


Figure 4.6 Presumed relationships between otters marked H, L, D and P, showing pairwise relatedness values, with LOD (log likelihood) scores for parentage analysis shown in italics underneath.

The second family group to be drawn using the genetic relatedness and parentage data involves otters H, L, D and P. Otters H and L share the highest pairwise relatedness value (r = 0.83) observed in this study and also share one of the highest parentage analysis scores (L.O.D. = 2.56). Together this suggests that otters H and L share a close genetic relationship, implying a parent-offspring or full sibling family bond.

Otters D and P are less closely related, but both have reasonably high (r = 0.34 - 0.45) levels of pairwise relatedness to both otters H and L, suggesting a more secondary family bond. It should be noted that otters D and P show a below average level of genetic relatedness to each other (r = -0.08), indicating they are unlikely to be related to each other.

As with the first inferred family, all four otters in this second group are located within a similar geographical area to each other (Figure 4.8). Otters H, L and P are all using the Rutherford tributary and its confluence with the main river. Otter D is observed just a short distance downstream of the confluence (Figure 4.8).



Otter	2005-2006								2006-2007								
	O	N	D	J	F	M	A	M	O	N	D	J	F	M	A	M	J
H					■												
L				▨					■			■					
D		■															
P															■		

Figure 4.7 Dates and locations for four otters that show significant ties of pairwise relatedness. Underneath is a chart showing months in which otters were sampled. Dark squares represent positives and the hatched square represents a find for which the genotypic assignment is poorly supported.

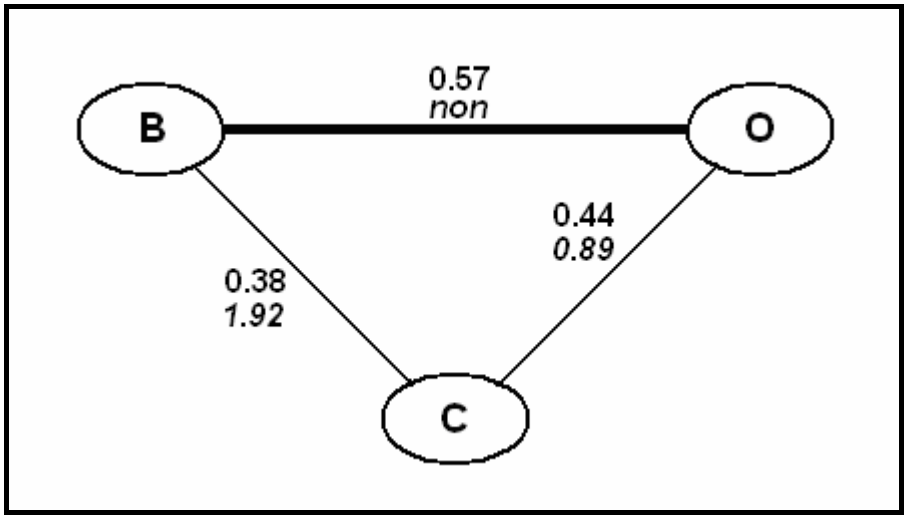
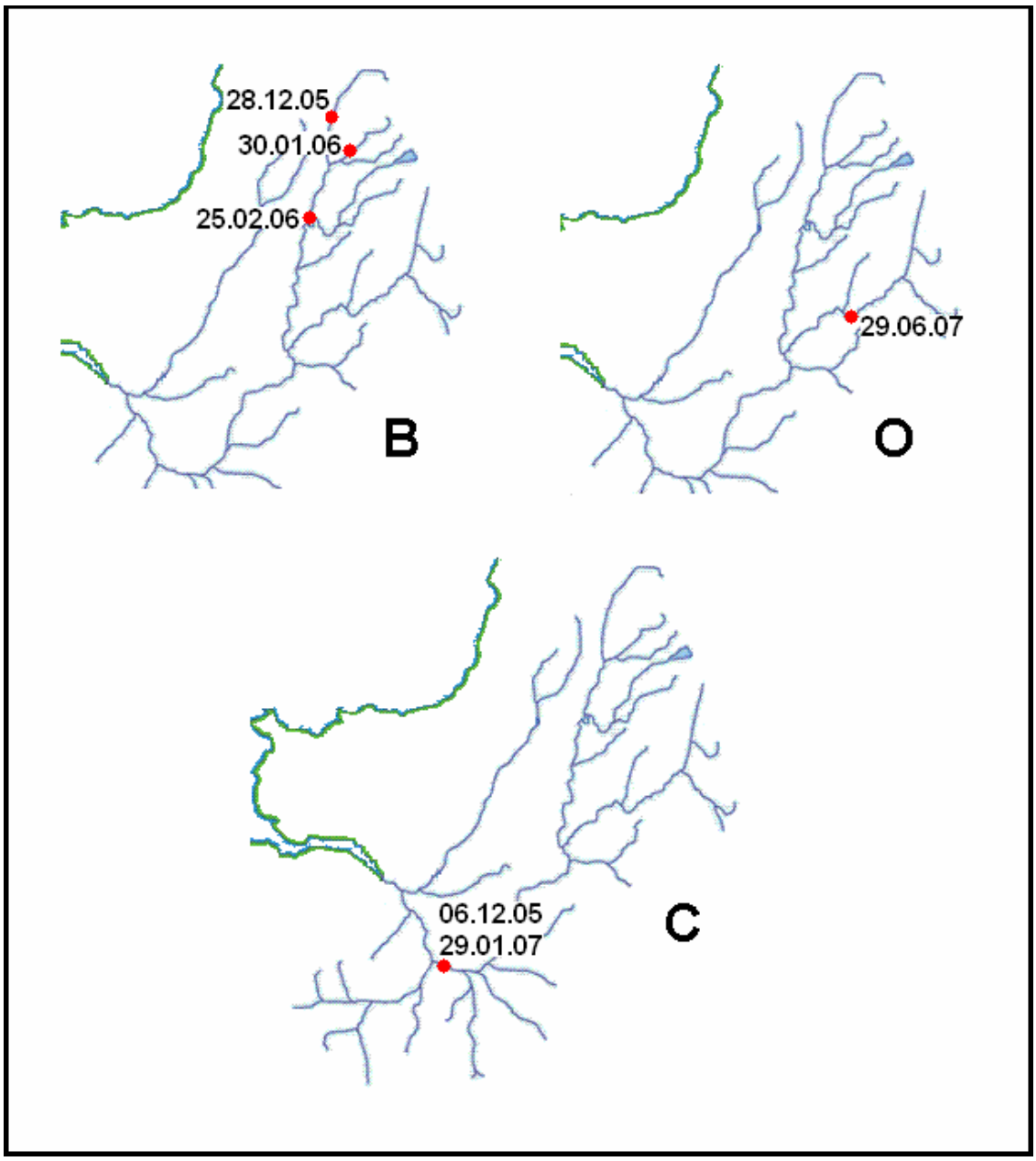


Figure 4.8 Presumed relationships between otters marked H, L, D and P, showing pairwise relatedness values, with LOD (log likelihood) scores for parentage analysis shown in italics underneath.

The third group inferred from relatedness and parentage values is that of otters B, C and O. Otters B and O share a high value of genetic relatedness (r = 0.57) but parentage analysis suggests that they are not related in a parent-offspring bond. It is likely that otters B and O are closely related, perhaps as full siblings. The third otter in the trio, otter C, shows relatively high values of relatedness and parentage with both otters B and O, although the values do not give much of an indication as to how close these relationships are likely to be.

Otters B and O were found to be using the upper reaches of the river. In contrast, otter C was found on the lower reaches, suggesting that, if these otters are genuinely related, then migration along the river system has separated members of this family group.

If some otters have not been detected, and their genotypes not identified, then some family groups and their relationships will be incomplete. Any migration of family members away from the river will also mean that some genetic ties may not have been identified.



Otter	2005-2006								2006-2007								
	O	N	D	J	F	M	A	M	O	N	D	J	F	M	A	M	J
B			■	■	■												
O																	■
C			■									■					

Figure 4.9 Dates and locations for three otters that show significant ties of pairwise relatedness. Underneath is a chart showing the months in which these otters were sampled. Dark squares represent positives.

4.2.4 Summary statistics for the River Camel samples

The River Camel otters show varying levels of heterozygosity at the ten loci analysed. In addition the number of alleles expressed per locus was generally low, with an average of 2.3 alleles per locus. Some loci were more informative than others. For example, locus 04OT14 expressed just two alleles, one of which was observed in only one individual and as a result heterozygosity was extremely low (H_O = 0.063), while locus 04OT07 expressed four different alleles and showed a relatively high level of heterozygosity (H_O = 0.786).

The River Camel spraint genotypes expressed variable levels of heterozygosity for the different loci. However, for nine out of the ten loci, heterozygosity (H_O) was equal to, or below, 0.5, indicating low levels of genetic diversity in the samples from the River Camel population. Similarly, the low number of alleles indicates a low level of genetic diversity.

Table 4.2 Summary statistics for the genotypes obtained from River Camel spraint samples. N = number of samples genotyped, A = number of alleles, He = expected heterozygosity, Ho = observed heterozygosity, HWE = Hardy-Weinberg equilibrium.

	N	A	He	Ho	HWE
Lut701b	16	2	0.500	0.250	0.054
Lut717b	16	2	0.498	0.438	0.638
04OT14	16	2	0.061	0.063	---
04OT17	16	2	0.305	0.250	0.434
Lut435	16	2	0.469	0.375	0.591
RIO11	16	2	0.305	0.375	1.000
04OT04b	12	2	0.469	0.417	1.000
04OT19	15	2	0.320	0.133	0.056
Lut832	16	3	0.580	0.500	0.054
04OT07	14	4	0.704	0.786	0.424

4.2.5 Comparisons with the wider Cornish population

The River Camel spraint genotypes were compared to the wider Cornish population, genotyped from tissue samples, to test the accuracy of the spraint genotypes (Broquet & Petit, 2004). If the genotypes obtained from the spraint material were inaccurate this would be seen as a large genetic differentiation between the spraint genotype data and the Cornish tissue genotype data.

This comparison can also demonstrate whether, or not, the River Camel population is typical of a Cornish otter population. In fact the level of genetic differentiation between the River Camel and Cornish otter populations was low (F_{ST} = 0.008), suggesting that the River Camel otter population is genetically representative of the wider Cornish population. It is important to remember that these two data sets are from two different time periods, different geographic areas and populations of different sizes, which may have biased the findings of the analysis.

4.3 Assessment of variation in genotyping success

One of the overall aims of the project was to improve and standardize the spraint genotyping technique and, as part of this, to improve DNA extraction and genotyping success rates. To this end, factors which may have influenced the likelihood of obtaining a genotype from a sample were investigated. Information collected in the field and observations made in the laboratory were used to try to identify factors that may inhibit or enhance the chances of successfully obtaining a genotype from a sample.

4.3.1 The influence of field based factors

Collection volunteers were provided with data sheets to record the date and location of sample collections and information on the conditions under which samples were collected (see section *2.1 Sample collection*). This information was used to investigate the effect that various factors had upon the likelihood of successfully obtaining a genotype. The six field factors included in the analysis were; sample type (anal jelly or spraint), weather condition (dry, wet or frost/snow), time of sample collection, level of sample exposure (exposed or protected), substrate under sample (stone, grass or other) and season (autumn = Oct – Dec, winter = Jan – Mar, and spring = Apr – Jun).

A logistic regression model was used, using the Forward:LR method, to extract from the list of six field factors those that have a significant effect on the likelihood of successfully obtaining a genotype. The model extracted spraint type ($X^2 = 7.967$, df = 1, $p = 0.005$) and weather ($X^2 = 6.854$, df = 2, $p = 0.032$) as having significant effects upon genotyping success rates. However, even after the inclusion of these two factors into the model the -2 log likelihood remained high (-2LL = 156.7), indicating that there is still a large amount of variation unaccounted for by the model.

The predicted probabilities produced by the model (Table 4.3) indicate that anal jelly samples are more likely to produce a genotype than spraint samples. Predicted probabilities are also higher for samples collected on days of snow or frost, suggesting that the colder conditions are more likely to result in successful DNA extraction and genotyping.

Table 4.3 Predicted probabilities produced by the logistic regression model of field collection conditions, predicting the likelihood that a sample possessing the stated characteristics will produce a genotype.

Sample type	Weather conditions	Predicted probabilities
Anal jelly	Dry	0.335
Anal jelly	Wet	0.331
Anal jelly	Snow/frost	0.652
Spraint	Dry	0.092
Spraint	Wet	0.091
Spraint	Snow/frost	0.275

It is noteworthy that for samples of spraint collected under dry or wet conditions, the successful acquisition of a genotype resulted in high standardized (ZRE) and studentized (SRE) residuals (ZRE = 3.136 and SRE = 2.195 for samples of spraint collected under dry conditions and ZRE = 3.164 and SRE = 2.216 for spraint collected under wet conditions). The high residuals indicate that the successful genotyping of these samples does not fit the model well and is further evidence of the poor overall fit of the model in terms of predicting which samples are likely to successfully produce a genotype.

In summary, of the six factors assessed, only sample type and weather conditions were shown to significantly affect the chances of successfully obtaining a genotype. However, these factors alone are not sufficient predictors of whether, or not, a particular sample will produce a genotype. In future studies samples of anal jelly and samples collected under cold weather conditions should be extracted and genotyped in preference to other samples, but samples should not be excluded from analysis purely on the basis of sample type or the weather conditions under which they were collected.

4.3.2 Sample observations and variation in success rates

Samples varied in terms of the amount and appearance of spraint material collected, and in the volume and appearance of the IMS collection medium the sample was stored in. Observations were made in the laboratory to try to assess whether any of these factors could be used to predict the likelihood of obtaining a genotype from a sample. This information may be useful in selecting the most productive samples for analysis in future studies. Only samples of spraint (i.e. not anal jelly) were included in the analysis of sample variation to eliminate any possible interaction effects between spraint type and the sample parameters being analysed. As the number of anal jelly samples analysed was small these samples were not subjected to further analysis.

Four factors were entered into the model: IMS colour (clear/pale or amber/dark), IMS:spraint ratio (poor, reasonable or good), spraint description (loose, silty or other) and spraint size (small, medium or large). The model extracted IMS colour (X^2 = 10.633, df = 1, p = 0.001) and IMS:spraint ratio (X^2 = 12.291, df = 2, p < 0.001) as having significant effects upon genotyping success rates. Together these factors account for 58.9% of the variation in the spraint sample data (remaining -2LL = 15.976).

Table 4.4 The predicted probabilities produced by the logistic regression model of spraint sample observations, predicting the likelihood that a sample possessing the stated characteristics will produce a genotype.

IMS colour	IMS:spraint ratio [a]	Predicted probabilities
Clear/pale	Poor	1.000
Clear/pale	Reasonable	0.083
Clear/pale	Good	0.167
Amber/dark	Poor	$2.5x10^{-9}$
Amber/dark	Reasonable	$8.2x10^{-19}$
Amber/dark	Good	$1.8x10^{-18}$

[a] ratios based on observational assessment of the volume of IMS in relation to the amount of spraint material collected

The predicted probabilities produced by the model (Table 4.4) indicate that, based on the results of this study, samples that turn the IMS amber or darker are extremely unlikely to produce a genotype. The predicted probabilities also suggest that samples stored in poor ratios of IMS:spraint are significantly more likely to produce a genotype. Based on these results it would be advisable for future studies to only extract samples held in poor ratios of clear or pale coloured IMS. It should be noted that these results are for samples stored in IMS and may not hold true for samples stored in the preferred biological grade ethanol medium.

4.4 Discussion - spraint genotyping analysis

4.4.1 Genotyping success rate

The cause of the poor success rates in obtaining genotypes from the River Itchen samples is not known. However, we can eliminate some factors that are unlikely to be relevant. Several of the volunteers collected spraint samples for a previous spraint genotyping project that is known to have produced successful genotypes (Coxon *et al.*, 1999; Dallas *et al.*, 2003). It is unlikely that the collection technique itself was at fault. Laboratory technique is unlikely to have been the cause of failure as the River Camel and River Itchen samples were treated in the same way once they reached the laboratory.

One possible cause of the poor success rates could have been the collection medium used. The medium in which the samples were collected and stored was changed several times throughout the project to try to ascertain whether this was the cause of the poor success rates. The last round of sample collections on the River Itchen used the same collection medium (IMS) and collection tubes as used on the River Camel. The success rates did not improve, despite standardising across the two sites, so it is unlikely that the collection medium accounted for the poor success rates.

Success rates for the River Camel samples were also low, but they were in line with success rates from other otter spraint genotyping studies (Coxon *et al.*, 1999; Dallas *et al.*, 2003; Arrendal *et al.*, 2007). Analysis of spraint collection data shows a significant link between spraint type and genotyping success rates; samples of anal jelly were significantly more likely to produce a genotype. Another factor that has a significant effect on genotyping success was the weather on the day of collection; samples collected in snow or frost were significantly more likely to produce a genotype than those collected in wet or dry weather. This suggests a correlation between the temperature on the day of collection and the likelihood of obtaining a genotype. Both of these factors are in agreement with results from previous otter spraint genotyping studies that report anal jelly samples are generally more likely to produce a genotype than spraint (Hung *et al.*, 2004; Arendal *et al.*, 2007), and samples collected in colder weather conditions are more likely to produce a genotype (Hájková *et al.*, 2006).

The anal jelly samples were dropped from the analysis, so as not to bias the results, and the spraint samples were analysed to see if the condition of the sample itself had any bearing on whether a successful genotype was obtained. The analysis showed IMS colouration had a significant effect. Samples that coloured the IMS amber, or darker, were significantly less likely to produce a genotype than those held in clear or lightly coloured IMS. The ratio of spraint:IMS also showed a significant link to genotyping success rates, with samples held in a poor ratio significantly more likely to produce a genotype. There are two possible reasons for this. Firstly, a smaller amount of IMS compared to the amount of spraint may result in a higher concentration of DNA in the IMS. Alternatively, the IMS may itself have a negative effect on the sample, in which case proportionally larger amounts of IMS might accentuate the effect on the sample. As the mechanism of the IMS effect is unknown it would be advisable for future otter spraint genotyping studies to use biological grade ethanol as a collection and storage medium. Ethanol is already commonly used in faecal genotyping studies (Frantzen *et al.*, 1998).

4.4.2 The River Camel study

The spraint genotyping technique was used to successfully genotype 41 samples from the River Camel catchment and these appear to represent 16 different otters. Combining the genotype information with collection information provided dates and locations for these 16 different otters. The otters were found in several distinct areas, with none of the otters using more than one of these areas. The data from this study suggests that the otters using the River Camel inhabit distinct home ranges with well defined boundaries. However, the relatively low number of observations, variation in sampling effort and the fact that half the otters were only observed once, means that more study is needed before definite conclusions can be made about home range usage.

Calculations of genetic relatedness revealed three potential family groups inhabiting three distinct areas of the river. This suggests that there is a healthy breeding population of otters using the River Camel. These relationship studies not only shed light on the relationship structure of the River Camel population, but also highlight the potential of this technique in future studies of otter behaviour.

5 Genetic population comparisons

One of the main aims of the study was to assess the genetic diversity of the River Itchen otter population and, in particular, to assess the likely causes of the genetic signature observed. Tissue samples were genotyped using eleven microsatellite loci, to compare the genetic diversity of samples from the River Itchen with those collected from the other study areas in Dorset and Cornwall. Samples from Dorset originated from an area along the coast to the west of the River Itchen. As with the River Itchen otter population, otter numbers in this area declined drastically at the time of national decline and, as in the River Itchen population, numbers were bolstered by the release of captive bred otters (Crawford, 2003). The Dorset population, being situated closer to the southwest otter population, was probably engulfed by population expansion from this area earlier than the River Itchen population. The Cornish otter population is situated in the heart of the southwest, a region identified as a stronghold for otters in England. It has suffered a less severe drop in numbers and, as a result, is likely to have retained much of its genetic diversity. Further information about the tissue samples analysed, e.g. the locations from which otter carcasses were collected, is given in Appendix 3.1.

Haplotype analysis of a region of the mitochondrial control region has been carried out for some of the otter populations around Europe (Mucci *et al.* 1999; Cassens *et al.* 2000; Ferrando *et al.* 2004; Pérez-Haro *et al.* 2005). In the current study, samples from England and Wales were analysed and the results were compared to those obtained from otters from the River Itchen population. This provides information on the genetic history of the otters currently inhabiting the River Itchen. In particular, the results provide an insight into the role that the release of captive-bred otters may have played in the recovery, and present day genetic make-up, of the Itchen otter population.

5.1 Microsatellite genotype analysis

The population genetic techniques used here are all standard methods for studying the genetic diversity within populations and the genetic differentiation between them. All of the population genetic calculations used in this study, including the calculation of Wright's Fixation index (F_{ST}), have been undertaken using standard population genetic methods. Details of these are readily available, see the following papers and books for further information: Hudson *et al.* (1992); Michalakis & Excoffier (1996); Beebee & Rowe (2008). It should be noted that the sample numbers used in this study would generally be considered too small for an accurate analysis to be carried out. This problem is inherent in studies of mammals, and in particular studies of top predators, such as the otter, where population numbers are relatively low.

5.1.1 Allele frequencies

Allele frequency data for the three populations sampled using DNA from tissue samples are presented in Appendix 3.2. Of note is the number of alleles which are not observed in all three populations. In particular, the number of alleles that are observed in only one of the three populations, known as private alleles, is relatively high. There are two from Cornwall, three from Dorset and four from the River Itchen. Due to the low sample numbers in the study, the number of private alleles may be an overestimate, particularly in the case of rare alleles. However, it is worth noting that the River Itchen population expressed the highest number of private alleles while the Cornish population expressed the lowest, despite the significantly larger number of samples from Cornwall (n = 16 versus n = 9 for the Itchen sample).

Also of note is the number of alleles that are only present in the River Itchen and Dorset samples (n = 7) and only present in the Cornish and Dorset samples (n = 5), versus just one allele that is present in the River Itchen and Cornish samples but not the Dorset samples. This suggests that the River Itchen and Cornish otter populations are more distinct from each other, in terms of allelic composition, than either population is from the Dorset population.

5.1.2 Allelic richness

Allelic richness is a measure of the number of alleles expressed in a population standardised for variation in sample numbers. For the data presented here, the method calculates the number of alleles that would have been found in the River Itchen and Cornish populations if only five otters had been sampled, as is the case in the Dorset population (Table 5.1). This allows comparison of the number of alleles in the three different populations, having taken into account differences in sample sizes.

The Dorset population shows the greatest level of allelic richness for the majority of microsatellite loci studied, while the Cornish population generally shows the lowest (Table 5.1). When averaged across the eleven loci, the Dorset population shows the highest level of allelic richness (A = 3.18), the Itchen the next highest (A = 2.78) and the Cornish population the lowest (A = 2.31).

5.1.3 Heterozygosity

A heterozygous individual is one that expresses two different allele sizes at a single locus. A measure of the heterozygosity in a population is the proportion of individuals that express two different allele sizes at the locus or loci in question. Levels of heterozygosity can be used to indicate genetic diversity - the greater the numbers of alleles and the more evenly spread they are, the more likely it is that high numbers of individuals are heterozygous and the more genetically diverse the population. Low levels of heterozygosity can indicate selective pressure on the population: for example, inbreeding will result in high numbers of individuals inheriting the same allele at a locus because their parents will be genetically more similar to one another than unrelated individuals.

Expected heterozygosity (He) is an estimate of the proportion of individuals expected to be heterozygous given the allele frequencies observed in a population. The observed heterozygosity (Ho) is the actual proportion of heterozygosity detected in a set of samples. The larger the difference between the observed and expected heterozygosity values, the greater the likelihood that there is some selective pressure causing the deviation from expected values. Hardy-Weinberg Equilibrium (HWE) probabilities measure the significance of any difference between expected and observed heterozygosity. In the genotype data the only locus to fall outside of HWE ($p < 0.05$) is locus 04OT19 in the Cornish population (Table 5.1). The locus is not outside of HWE in the other two populations. This suggests that the deviation from HWE is not caused by selective pressure on the locus itself and therefore, it is not necessary to exclude the locus from further analyses.

Observed heterozygosity, measured across all eleven loci, suggests that the Dorset population was the most genetically diverse population surveyed (Ho = 0.509), while the Cornish population was the least diverse (Ho = 0.344). The heterozygosity of the River Itchen population lies between the other two (Ho = 0.415) and shows the Itchen to be relatively diverse. The results demonstrate the reverse of what would be expected if sample size was affecting the results. The Dorset population shows the highest heterozygosity, but the lowest sample number and *vice versa* for the Cornish population.

5.1.4 Linkage disequilibrium

Three pairs of loci show significant linkage disequilibrium; loci 04OT04 and 04OT07 in the Cornish population ($p = 0.043$), loci Lut717 and Lut435 in the River Itchen population ($p = 0.005$), and loci 04OT17 and Lut832 in the River Itchen population ($p = 0.049$). In each case the linkage disequilibrium is only significant in one population, suggesting that the loci are not physically linked to each other in the genome.

Table 5.1 Summary statistics per microsatellite locus, per population A = allelic richness standardised for the minimum number of individuals in a population (n = 5), He = expected heterozygosity, Ho = observed heterozygosity, HWE = HWE probabilities.

	Lut701b	Lut717b	04OT14	04OT17	Lut435	RIO11	04OT04b	04OT19	Lut832	04OT07	RIO18
Cornwall (n = 16)											
A	2.00	2.31	1.53	1.69	2.53	1.98	2.00	1.99	3.70	4.12	1.53
He	0.492	0.506	0.117	0.170	0.549	0.404	0.482	0.430	0.704	0.744	0.117
Ho	0.500	0.313	0.125	0.188	0.438	0.438	0.563	0.125	0.533	0.438	0.125
HWE	1.000	0.099	1.000	1.000	0.290	1.000	1.000	0.008**	0.379	0.062	1.000
River Itchen (n = 9)											
A	2.99	2.56	3.63	2.99	2.93	2.75	1.99	2.00	3.79	3.93	1.00
He	0.656	0.537	0.667	0.648	0.586	0.438	0.401	0.494	0.710	0.741	0.000
Ho	0.625	0.556	0.333	0.444	0.444	0.444	0.333	0.222	0.667	0.889	0.000
HWE	0.511	0.723	0.052	0.182	0.134	0.530	1.000	0.172	0.408	0.496	-----
Dorset (n = 5)											
A	4.00	3.00	1.00	2.00	3.00	3.00	4.00	3.00	5.00	5.00	2.00
He	0.700	0.660	0.000	0.480	0.620	0.640	0.580	0.560	0.680	0.680	0.180
Ho	0.800	0.600	0.000	0.400	0.800	0.400	0.400	0.600	0.800	0.600	0.200
HWE	0.696	0.544	-----	1.000	1.000	0.113	0.240	1.000	0.483	0.228	-----

** = significantly deviated from HWE, $p < 0.01$

5.1.5 Genetic differentiation

The statistical calculation of genetic differentiation (F_{ST}) is used to estimate the level of genetic similarity between two populations. This can then be used to assess how geographic and demographic factors affect the genetic distinctiveness of populations in relation to each other. F_{ST} estimates indicate that the most genetically differentiated populations were Cornwall and the River Itchen, whilst the most genetically similar populations were the River Itchen and Dorset populations (Table 5.2).

Table 5.2 Genetic differentiation (F_{ST}) between pairs of populations.

	Cornwall	River Itchen
River Itchen	0.189	
Dorset	0.128	0.034

5.1.6 Allele-sharing

Phylogenetic trees use genetic information to infer the evolutionary relationship between populations or species. One commonly used approach is the neighbour-joining method in which trees are constructed using the principles of minimum evolution or maximum parsimony (Saitou & Nei, 1987): these methods work on the principal that evolution acts by the simplest or shortest route. The information from microsatellite genotypes can be used to construct a phylogenetic tree where the levels of allele sharing between genotypes are used as a measure of genetic distance. These genetic distances are then used to calculate the relationships between operational taxonomic units (OTUs). OTUs can be populations (Kyle & Strobeck, 2001), groups of individuals within a population or, as in this study, individual animals (Estoup *et al.*, 1995; Edwards *et al.*, 2000; Tapio *et al.*, 2005; Rengmark *et al.*, 2006). In the resulting tree the branch lengths represent genetic distances and the separation between the branches represents the level of genetic separation between samples.

The allele-sharing, neighbour-joining tree constructed using the River Itchen, Dorset and Cornish samples (Figure 5.1), shows a definite grouping of genotypes derived from the Cornish otter population; all the Cornish samples are found within one main branch of the tree. With a couple of exceptions that group with the Cornish samples (samples M1053 and M1064), otters collected from the River Itchen and Dorset are positioned on a separate section of the tree. This Itchen/Dorset clade is less compact than the Cornish portion of the tree and the longer branch lengths indicate greater genetic differentiation between the individuals. Otters from Dorset are admixed with otters from the River Itchen, suggesting that the two populations are genetically similar to each other.

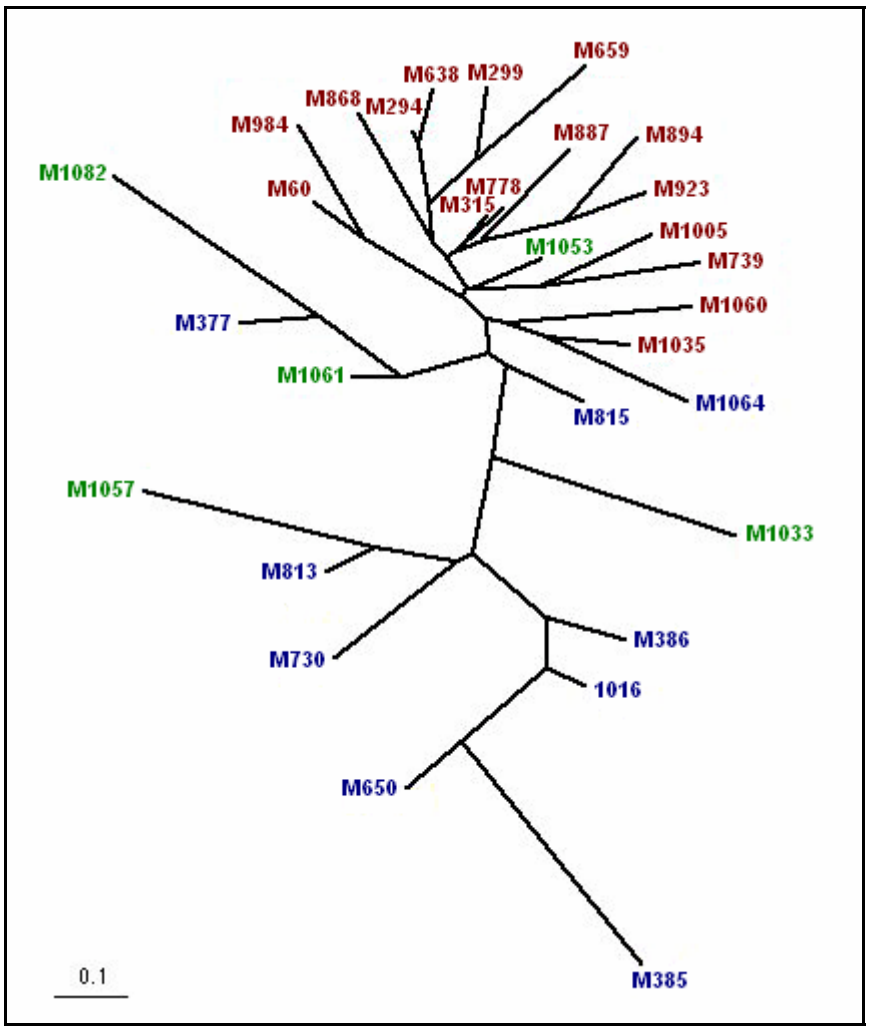


Figure 5.1 An allele-sharing, neighbour-joining tree representing individuals from the three populations; the Cornish population is shown in red, the River Itchen population in blue, and the Dorset population in green.

5.2 MtDNA haplotype analysis

5.2.1 Background

This study was conducted to investigate to what extent captive bred otters, released onto the River Itchen in the early 1990s, have integrated into the wild otter population. The aim was to add to our wider understanding of the genetic effects of the release programme on resident otter populations, such as that of the River Itchen. The 5´ end of the mitochondrial control region was analysed in otters from four locations across England and Wales. This section of DNA, roughly 300bp in length, has been used successfully in a number of previous studies (Mucci *et al.* 1999; Cassens *et al.* 2000; Pérez-Haro *et al.* 2005) to characterise otter haplotypes from populations across Europe, but as yet little work has been carried out on otters in England and Wales (Ferrando *et al.* 2004; Stanton *et al.*, 2008).

Across Europe the haplotype Lut1 dominates, with isolated occurrences of haplotype variation (Figure 5.2). It has been suggested that this pattern reflects glacial refugia during the Pleistocene period (Somme & Benecke, 2004; Somme & Nadachowski, 2006). Otters were unable to survive across much of Europe during the glacial period and only populations in isolated areas of southern Europe survived. It is likely that the haplotype Lut1 became fixed in one of these small isolated populations and subsequently spread across Europe as the otter population moved out from the refuge after the last Ice Age. The solitary instances of haplotype variation are likely to have originated from more recent mutational events spreading through local populations. The exception to this is the haplotype Lut3, which is found in a high proportion of otters sampled from eastern Germany and in one of three otters sampled from Belarus. Given the geographical separation of these sites (Figure 5.2) this suggests that either two independent mutational events occurred to produce the same haplotype or that otters with this haplotype spread from a second refuge in Eastern Europe.

The most comprehensive study to date found a significantly greater level of variation in English and Welsh otter populations than is found in other European countries (Stanton *et al.*, 2008). In otters sampled from Wales and western England (n = 45) the haplotype Lut6 was the most common haplotype, occurring in 53% of the otters sampled. The haplotype Lut7 was also common in this area, found in 27% of otters; the remaining 20% of otters expressed the haplotype Lut1. The fact that neither haplotypes Lut6 or Lut7 have been found in any other wild European population suggests that these haplotypes are native to western Britain and are likely to be the result of mutational events in otters in this area. In samples collected from eastern England, Stanton *et al.* (2008) found that 12 out of the 13 otters sampled expressed the Lut3 haplotype, the remaining otter expressed the haplotype Lut1.

Records are sporadic, but well over 100 otters were released from the Otter Trust captive breeding programme. The majority of these were released onto rivers in East Anglia, the Thames Valley and Yorkshire (Crawford, 2003; The Otter Trust, 2006). After the releases, otter numbers in these regions rose sharply (Crawford, 2003) suggesting that the population in the east of England today contains a high proportion of otters descendant from the captive breeding programme. Previous studies of otters descendant from 'English breeding stock' have found a high instance of the Lut3 haplotype (Mucci *et al.* 1999; Pérez-Haro *et al.* 2005; realignment of sequences from Ketmaier & Bernardini, 2005), with only one family of otters found to express the haplotype Lut6 (Pérez-Haro *et al.* 2005). This, along with the prevalence of the Lut3 haplotype in otters from eastern England, suggests that the Lut3 haplotype may legitimately be considered synonymous with the Otter Trust captive breeding programme.

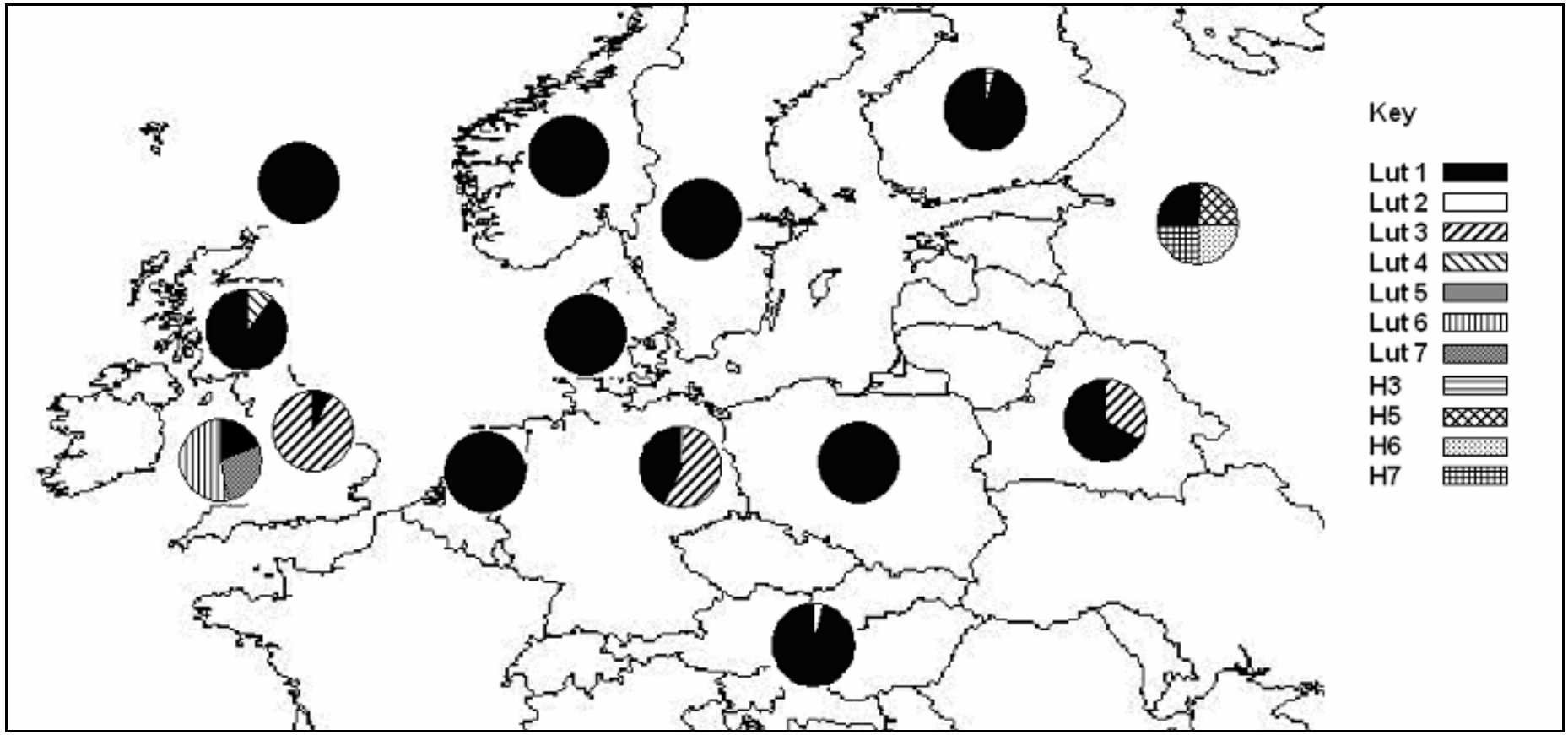


Figure 5.2 Haplotypes of otters from across northern Europe. Data amalgamated from Mucci et al. (1999), Cassens et al. (2000), Ferrando et al. (2004) and Pérez-Haro et al. (2005), Stanton et al. (2008). Sample sizes as follows: Shetland (4); Scotland (19); Wales and Western England (45); Eastern England (13); Finland (26); Norway (4); Sweden (5); Denmark (30); Holland (5); Eastern Germany (76); Poland (1); Belarus (3); Russia (4) and Austria and Hungry (25).

5.2.2 Results from this study

As some of the samples analysed in this study are the same as those analysed by Stanton *et al.* (2008) it is perhaps not surprising that similar haplotype profiles were obtained. In contrast to the previous study, our analysis was carried out at a more local scale, providing greater separation between locations.

Our analysis shows that the dominant haplotype in Cornwall is Lut1, all eleven samples from this region expressed this haplotype. In Wales, the haplotypes Lut1, Lut6 and Lut7 are all present. The Cornish and Welsh otter populations are not thought to have received captive bred otters and the haplotypes observed in these regions can be considered to be native to their respective areas. The haplotypes Lut6 and Lut7 have only been observed in the Welsh population and it is likely that these haplotypes have arisen in this geographical region through mutational events.

Of the seven otters sampled from East Anglia six expressed the haplotype Lut3, the remaining otter expressing the Lut1 haplotype. This is in line with findings by Stanton *et al.* (2008), where Lut3 was the major haplotype observed in the populations of eastern England. Our results support the claim that the haplotype Lut3 is likely to be a signature haplotype of the captive bred otters.

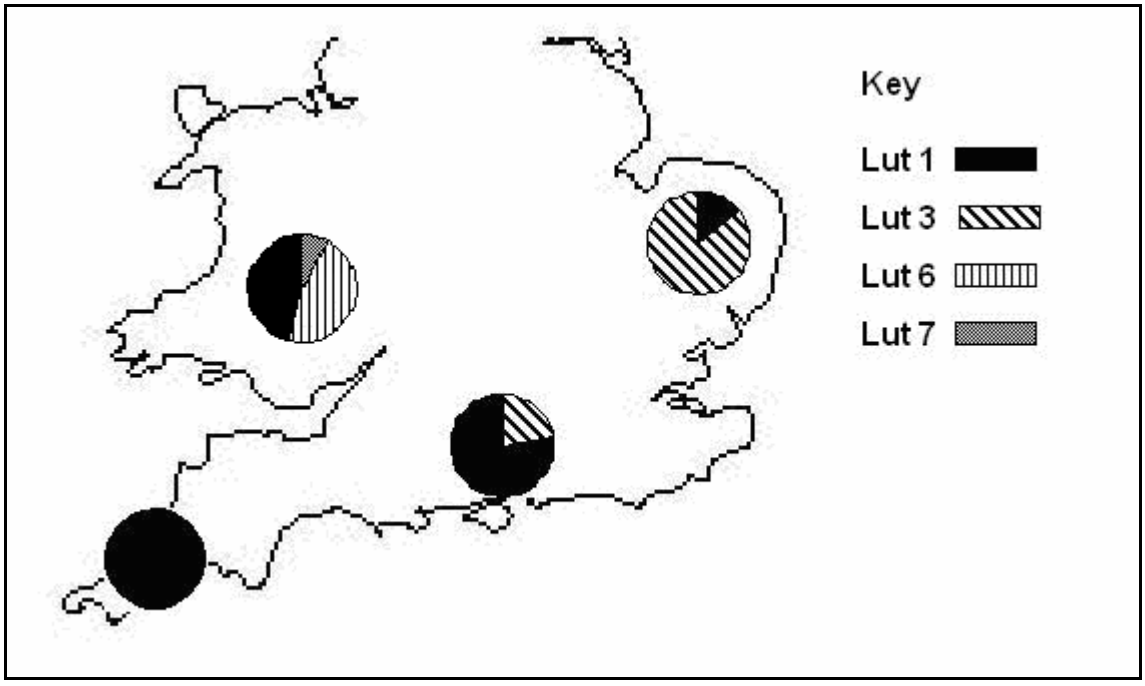


Figure 5.3 Haplotypes from the four British locations analysed in this study: Wales (13), East Anglia (7), Cornwall (11) and the River Itchen in Hampshire (9).

Of particular interest is the River Itchen otter population where, of the nine otters analysed, seven expressed the haplotype Lut1 and the remaining two expressed the haplotype Lut3. The high proportion of otters expressing the Lut1 haplotype, combined with the fact that all otters sampled from the Cornish population expressed this haplotype, suggests that this haplotype is the main, if not the only, haplotype expressed in the native otter population of southwest England. However, in the River Itchen population the presence of the Lut3 haplotype is of particular interest. Without sampling all of the otters from before and after the release programme it is not possible to be certain, however, given what we know about the link between captive bred otters and the Lut3 haplotype, the presence of the Lut3 haplotype in the River Itchen population strongly suggests that descendants of the captive bred otters are surviving in the current population. The presence of the Lut3 haplotype in the River Itchen populations can be taken as evidence that the captive released otters have bred within the River Itchen otter population.

It is worth noting that the haplotype is situated in the control region of the mitochondrial DNA and therefore is only passed down the maternal genetic line. This means that the signature of any male captive released otters will not show when using this technique. As the Lut3 haplotype is only passed on by females, the signature will be lost whenever males that express the haplotype breed (Figure 5.4). Consequently, the results of this study may underestimate the proportion of the extant River Itchen otter population that is descendant from captive-bred stock.

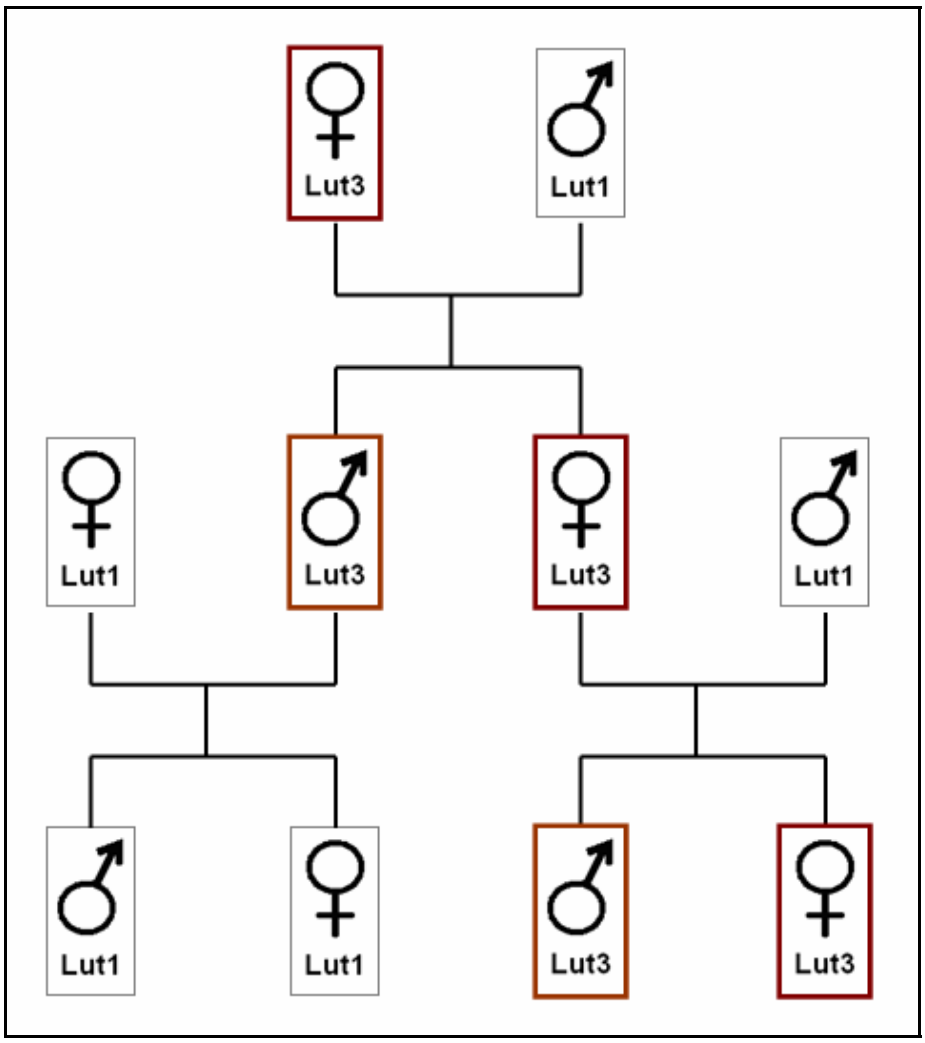


Figure 5.4 Mitochondrial haplotypes, like the one analysed in this study, are passed on from mother to offspring. This means that males with a particular haplotype will not pass it on to the next generation when they breed.

5.3 Discussion - the River Itchen otter population

The main focus of this project was to assess the genetic health and history of the River Itchen population by carrying out comparisons with other English and Welsh otter populations with varying demographic histories. Two main methods were used to carry out the comparisons; microsatellite genotype analysis was used to look at the genetic diversity of populations and mitochondrial haplotype analysis was used to assess the genetic origin of the otters present in the River Itchen population.

The first comparison used microsatellite genotype analysis to compare the River Itchen population to the neighbouring Dorset population and the Cornish population. The Cornish otter population suffered a much lower reduction in numbers during the period of national population decline and to our knowledge received no captive released otters, thereby making it an ideal population for comparison. The Dorset population on the other hand went through a similar population decline and reintroduction programme as the River Itchen, albeit to a lesser extent.

Otters from the River Itchen, Dorset and eastern Cornish populations were genotyped using a suite of eleven microsatellite loci to compare the genetic compositions of the three populations. Analysis of allelic composition and genetic differentiation statistics (F_{ST}) indicates that the River Itchen population is genetically more similar to the Dorset population than to the Cornish otter population. An allele-sharing, neighbour-joining tree shows a similar split, with genotypes of otters from the River Itchen and Dorset populations generally sitting on one branch of the tree and the Cornish otters grouped on a second, more tightly packed branch. This pattern fits expectations based on the geographical separation between populations; the Dorset population is far closer to the River Itchen in terms of geography as well as genetics.

Perhaps more striking is that the River Itchen otter population appears to be more genetically diverse than the Cornish population, as is evident when looking at allelic richness and heterozygosity data. This is the opposite of what would be expected when considering the population bottleneck that occurred on the River Itchen during the decline in otter numbers and the relatively healthy status of the Cornish population during the same period (Crawford, 2003). The high genetic diversity observed in the River Itchen population has probably resulted from the introduction of genetic material from external sources, the most likely explanation being the release of captive bred otters into the population in the early 1990s. Of the three populations studied using microsatellite analysis, the Dorset population was the most genetically diverse, suggesting that it too received genetic input from released captive bred otters. It may also have benefited from its position close to the leading edge of the otter population that is expanding from southwest England.

The sample numbers used in the genotyping study would normally be considered too small to provide accurate population comparisons. However, the populations with the smallest sample sizes are those which show the greatest genetic diversity. This is the opposite of what would be expected if sample numbers were truly a problem. It is possible that the trends we observed would have been even stronger if more samples had been analysed.

The second comparison was of haplotypes from otters from areas across England and Wales. This established the haplotype signature of the 'natural' population and of released captive bred otters. The objective was to establish whether the otters released from the captive breeding programme had bred with the resident otters on the River Itchen. A significant proportion (22%) of the otters sampled from the River Itchen site were found to express the haplotype Lut3, this is the same haplotype expressed by the majority of the English captive bred otters, as identified here and in previous studies (Mucci *et al.* 1999; Pérez-Haro *et al.* 2005; Ketmaier & Bernardini, 2005).

The haplotype analysis, in particular the presence of the Lut3 haplotype, indicates that captive bred otters released onto the River Itchen in the early 1990s have indeed bred within the local population. This raises questions over the integrity of the Itchen population, particularly as the origins of the otters used in the Otter Trust captive breeding programme are largely unknown. The apparent increase in genetic diversity, as evidenced by the microsatellite genotype analysis of the River Itchen and Cornish populations, could be considered advantageous if increased diversity is equated with an increased health of the population. However, great care must be taken when

making this assumption. If the otters used in the captive breeding programme originated from a source too genetically distinct from the River Itchen population, this may result in a phenomenon known as outbreeding depression (Lynch *et al.*, 1991; Michaux *et al.*, 2004), where the incompatibility of genes from the two sources can have negative effects on the health of individuals and ultimately the population itself.

6 PBDE concentrations

PBDE commercial mixtures have been used as flame retardants in many manmade materials, including textiles (not clothing), building materials, polystyrene and in the outer casings and internal components of electrical equipment such as TV sets, computer equipment and household appliances (Hooper *et al.*, 2004; BSEF, 2006). Bans on the manufacture and use of PBDEs within the European Union have recently come into effect (The European Parliament and the Council of the European Union, 2003; BSEF, 2008). The use and disposal of materials containing PBDEs that were produced before the ban came into effect, combined with the environmental persistence of PBDEs, means that PBDEs are likely to be an environmental issue of concern for years to come.

The basic structure of polybrominated diphenyl ethers is two hydrocarbon rings linked by an oxygen molecule (Figure 6.1). The individual PBDE congeners are defined by the number and location of bromine molecules surrounding the central diphenyl ether structure. This means that there are 209 possible congeners, ranging from mono-BDEs, with one bromine molecule, to BDE-209 or deca-BDE, with ten bromines surrounding the central BDE structure.

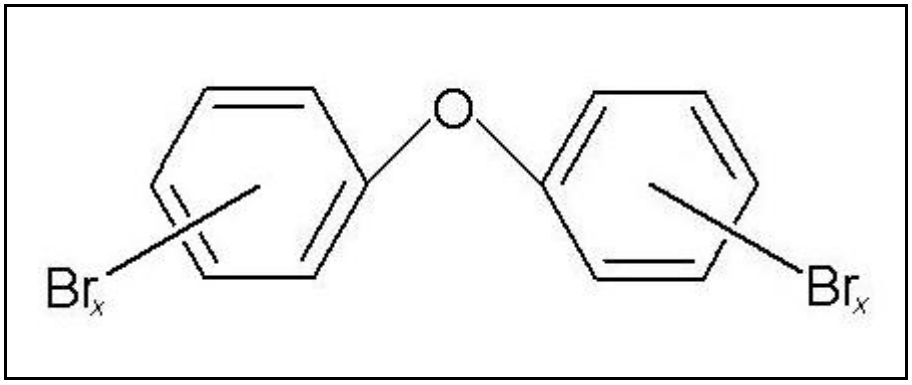


Figure 6.1 The basic structure of BDE congeners; showing the diphenyl ether structure surrounded by bromine molecules, the number and location of which defines the congener.

Some PBDE congeners are more easily synthesised than others and so these are found in the highest concentrations in commercial mixtures. There are three commercial PBDE mixtures: Penta-BDE, containing mostly tetra- and penta-BDE congeners (i.e. those congeners with 4 or 5 bromine molecules); Octa-BDE, containing hexa- to nona-BDE congeners (6 to 9 bromines) and Deca-BDE, consisting primarily of the largest congener BDE-209 (Table 6.1). The PBDE congeners that are found at the highest concentrations in the environment, specifically BDE-47 (tetra-BDE), -99, -100 (penta-BDEs), -153 (hexa-BDE) and -209 (deca-BDE), are those that are found in high concentrations in the commercial mixtures. Once in the environment the lower congeners (those with fewer bromines) tend to be more mobile and as a result tend to leach into freshwater and marine ecosystems. Higher congeners, in particular octa- to deca-BDEs, are more likely to become associated with the particulate matter of soils and sediments.

Table 6.1 The proportions of each BDE commercial mixture made up of the different sized congener groups, tri-BDE with three bromines to deca-BDE with ten. Data are taken from Darnerud *et al.* (2001), originally from IPCS (1994).

Congener group	Commercial mixture		
	Penta-BDE (%)	Octa-BDE (%)	Deca-BDE (%)
Tri-BDE	0-1		
Tetra-BDE	24-38		
Penta-BDE	50-62		
Hexa-BDE	4-8	10-12	
Hepta-BDE		43-44	
Octa-BDE		31-35	
Nona-BDE		9-11	0.3-3
Deca-BDE		0-1	97-98

PBDEs, as single congeners and as commercial mixtures, have been shown to induce several potential toxicological effects. The ability of PBDEs to suppress levels of thyroxine (T_4), a hormone essential for the regulation of growth and development, has been proved in laboratory studies (Zhou *et al.*, 2001; Hallgren *et al.*, 2001; Hallgren & Darnerud, 2002) and via biological sampling of wild populations (Hall *et al.*, 2003; Fernie *et al.*, 2005). This effect can be related to a string of other negative effects, such as a reduction in vitamin A (retinol) concentrations (Simpson *et al.*, 2000) and an increase in liver somatic indexes (Darnerud *et al.*, 2001; Zhou *et al.*, 2001). Exposure to PBDEs has also been linked to neurotoxic effects. For example, rats and mice exposed to BDE-99 or BDE-209 during early stages of development, in the womb or shortly after birth, exhibit significant deviations in several behavioural parameters later in life (Viberg *et al.*, 2003; Viberg *et al.*, 2004; Branchi *et al.*, 2005).

The toxicological effects of PBDEs may not to be as severe as those of PCBs (Hallgren *et al.*, 2001; Hallgren & Darnerud, 2002), but they are still a cause for concern when animals are exposed to them. As the potential for additive and synergistic effects in animals exposed to a mixture of organic pollutants is not fully understood, it is possible that PBDE contaminants may increase the negative effects of other, more toxic, pollutants (Hallgren & Darnerud, 2002).

6.1 PBDE congener concentrations

6.1.1 Congener profile

The concentrations of individual PBDE congeners found in otter liver tissue can be viewed in Table 6.2. Concentrations are presented relative to both the wet weight and lipid content of the liver tissue. The congener profile of the English and Welsh population of European otters was dominated by the BDE-47 congener, which on average was 71.5% of the total (∑) BDE concentration. The two next most common congeners, BDE-153 and BDE-209, were found at considerably lower concentrations:

8.8% (BDE-153) and 8.2% (BDE-209) of the ∑BDE concentration, respectively. A small but significant proportion of the congener profile comprised the congeners BDE-100 (4.0%), BDE-99 (1.4%) and BDE-207 (2.9%).

Table 6.2 PBDE congener concentration statistics for otter liver samples (n = 129) collected from locations across England and Wales.

Congener	Concentration in ng.g^{-1} wet weight			Concentration in ng.g^{-1} lipid weight		
	Mean	Median	Range	Mean	Median	Range
BDE-17	0.008	L.O.D.	<0.01-0.226	0.211	L.O.D.	<0.1 - 7.125
BDE-28	0.442	0.066	<0.04 - 39.01	9.138	1.965	<0.4 - 735.82
BDE-32	0.000	L.O.D.	<0.01 - 0.017	0.007	L.O.D.	<0.1 - 0.355
BDE-35	0.035	L.O.D.	<0.01 - 0.608	1.011	L.O.D.	<0.1 - 30.00
BDE-37	0.004	L.O.D.	<0.01 – 0.182	0.081	L.O.D.	<0.1 - 3.004
BDE-47	84.325	38.138	<1.1 - 1512.3	2228.527	1125.346	<10.4 - 34610.6
BDE-66	0.197	L.O.D.	<0.01 - 6.607	5.251	L.O.D.	<1.2 - 124.62
BDE-71	0.056	L.O.D.	<0.01 - 0.879	1.553	L.O.D.	<0.1 - 18.97
BDE-77	0.227	L.O.D.	<0.01 - 19.66	4.357	L.O.D.	<0.8 - 239.06
BDE-85	0.366	0.121	<0.04 - 16.52	8.173	3.312	<0.5 - 311.69
BDE-99	2.470	0.548	<0.1 - 137.62	51.009	15.591	<1.5 - 2595.77
BDE-100	4.857	1.845	<0.2 - 159.73	122.366	51.014	<2.5 - 3012.87
BDE-119	0.010	L.O.D.	<0.02 - 1.261	0.276	L.O.D.	<0.2 – 35.39
BDE-128	1.886	L.O.D.	<0.2 - 210.20	37.155	L.O.D.	<2.7 – 3964.8
BDE-138	1.486	0.173	<0.04 - 141.84	30.687	4.954	<0.7 – 2675.4
BDE-153	19.013	3.217	<0.3 - 1236.2	447.530	96.712	<5.9 – 23317.5
BDE-154	0.494	0.092	<0.03 - 39.73	10.341	2.375	<0.8 – 749.44
BDE-166	0.013	L.O.D.	<0.02 - 0.937	0.406	L.O.D.	<0.2 – 34.77
BDE-183	0.010	L.O.D.	<0.04 - 0.177	0.294	L.O.D.	<0.4 – 7.004
BDE-190	1.360	L.O.D.	<0.1 - 172.95	25.590	L.O.D.	<1.4 – 3262.2
BDE-196	0.004	L.O.D.	<0.05 - 0.241	0.075	L.O.D.	<0.5 – 3.937
BDE-197	0.271	L.O.D.	<0.06 - 31.63	5.309	L.O.D.	<0.6 – 596.53
BDE-206	0.086	0.045	<0.01 – 1.367	2.137	1.210	<0.3 – 24.90
BDE-207	1.381	0.753	0.080 – 30.85	39.576	19.321	1.526 – 858.45
BDE-208	0.254	0.146	<0.01 – 3.608	7.349	3.597	<0.17 – 101.16
BDE-209	5.852	2.252	<0.3 – 246.13	167.318	62.246	<8.5 – 6808.1
∑BDE	126.850	53.582	0.18 - 3705.0	3241.943	1564.483	12.18 – 69882.5

6.1.2 Congener correlations

The PBDE congener concentrations present in environmental samples, including the otter liver samples analysed in this study, are likely to have originated from just a few distinct sources, i.e. from the three commercial mixtures. As the BDE congeners have originated from PBDE commercial mixtures, this should be reflected in their relative concentrations in the otter liver samples. If this is the case, then, for example, an otter exposed to high concentrations of the Penta-BDE mixture would be expected to contain particularly high concentrations of congeners BDE-47, -99 and -100, all of which originate from this commercial mixture.

A matrix of Pearson's correlation coefficients is shown in Table 6.3. The closer a value is to one (or minus one) the greater the correlation between the concentrations of the two congeners. As an example, two congeners showing high levels of correlation were BDE-207 and BDE-209 (correlation coefficient of 0.960). The correlation can be easily seen in a scatter-plot of concentrations, where high concentrations of one congener generally correspond with high concentrations of the other (Figure 6.2). Pairs of congeners with concentrations that show low levels of correlation (Figure 6.3) have correlation coefficients close to zero. The correlation coefficient matrix demonstrates which groups of congeners are correlated with each other.

The correlation matrix (Table 6.3) shows that there is a significant correlation between twelve of the most concentrated tri- to octa-BDE congeners, suggesting that these congeners all originate from the same source. Note that the congeners BDE-47, -66 and -100 have lower correlation values than do other congeners in this set of correlated congeners. A second group of congeners highlighted by the matrix correlates BDE-196 with the nona- and deca-BDE congeners. This suggests that this group of congeners probably originate from the same sources.

Table 6.3 Pearson's correlation matrix for 18 PBDE congeners measured in otter liver tissue samples collected from across England and Wales. Highlighted values are significant at p <0.01.

	BDE -28	BDE -47	BDE -66	BDE -85	BDE -99	BDE -100	BDE -128	BDE -138	BDE -153	BDE -154	BDE -183	BDE -190	BDE -196	BDE -197	BDE -206	BDE -207	BDE -208	BDE -209
BDE-28	**1.000**																	
BDE-47	**0.609**	**1.000**																
BDE-66	**0.708**	**0.610**	**1.000**															
BDE-85	**0.911**	**0.558**	**0.656**	**1.000**														
BDE-99	**0.935**	**0.559**	**0.692**	**0.970**	**1.000**													
BDE-100	**0.821**	**0.598**	**0.604**	**0.910**	**0.867**	**1.000**												
BDE-128	**0.995**	**0.550**	**0.715**	**0.916**	**0.941**	**0.802**	**1.000**											
BDE-138	**0.993**	**0.549**	**0.720**	**0.924**	**0.944**	**0.802**	**0.997**	**1.000**										
BDE-153	**0.951**	**0.575**	**0.678**	**0.902**	**0.903**	**0.796**	**0.995**	**0.951**	**1.000**									
BDE-154	**0.989**	**0.559**	**0.703**	**0.950**	**0.961**	**0.839**	**0.993**	**0.994**	**0.959**	**1.000**								
BDE-183	-0.029	-0.071	0.047	-0.053	-0.049	0.003	-0.028	-0.030	-0.053	-0.036	**1.000**							
BDE-190	**0.995**	**0.544**	**0.707**	**0.916**	**0.942**	**0.800**	**0.999**	**0.998**	**0.951**	**0.994**	-0.027	**1.000**						
BDE-196	-0.016	-0.041	0.001	-0.034	-0.024	-0.035	-0.014	-0.018	-0.025	-0.020	0.083	-0.015	**1.000**					
BDE-197	**0.994**	**0.543**	**0.707**	**0.918**	**0.942**	**0.800**	**0.999**	**0.997**	**0.951**	**0.994**	-0.025	**0.999**	0.011	**1.000**				
BDE-206	-0.036	0.199	0.173	-0.050	-0.035	-0.011	-0.043	-0.052	-0.009	-0.055	0.095	-0.052	**0.681**	-0.029	**1.000**			
BDE-207	-0.032	0.081	0.103	-0.036	-0.037	-0.038	-0.034	-0.041	-0.018	-0.040	0.053	-0.040	**0.740**	-0.005	**0.834**	**1.000**		
BDE-208	-0.043	0.177	0.191	-0.054	-0.037	-0.050	-0.049	-0.056	-0.021	-0.056	0.080	-0.050	**0.575**	-0.026	**0.857**	**0.921**	**1.000**	
BDE-209	-0.017	0.029	0.065	-0.029	-0.028	-0.029	-0.020	-0.024	-0.017	-0.027	0.080	-0.023	**0.821**	0.009	**0.796**	**0.960**	**0.833**	**1.000**

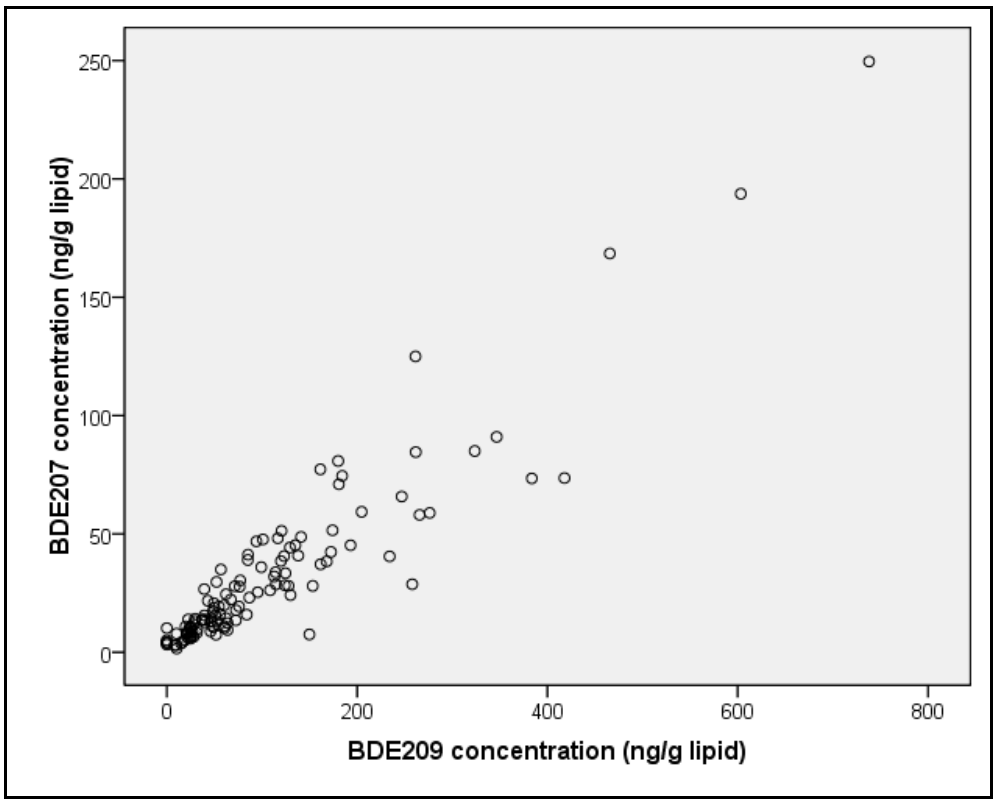


Figure 6.2 A positive correlation between relative concentrations of BDE-207 and BDE-209 in otter liver samples.

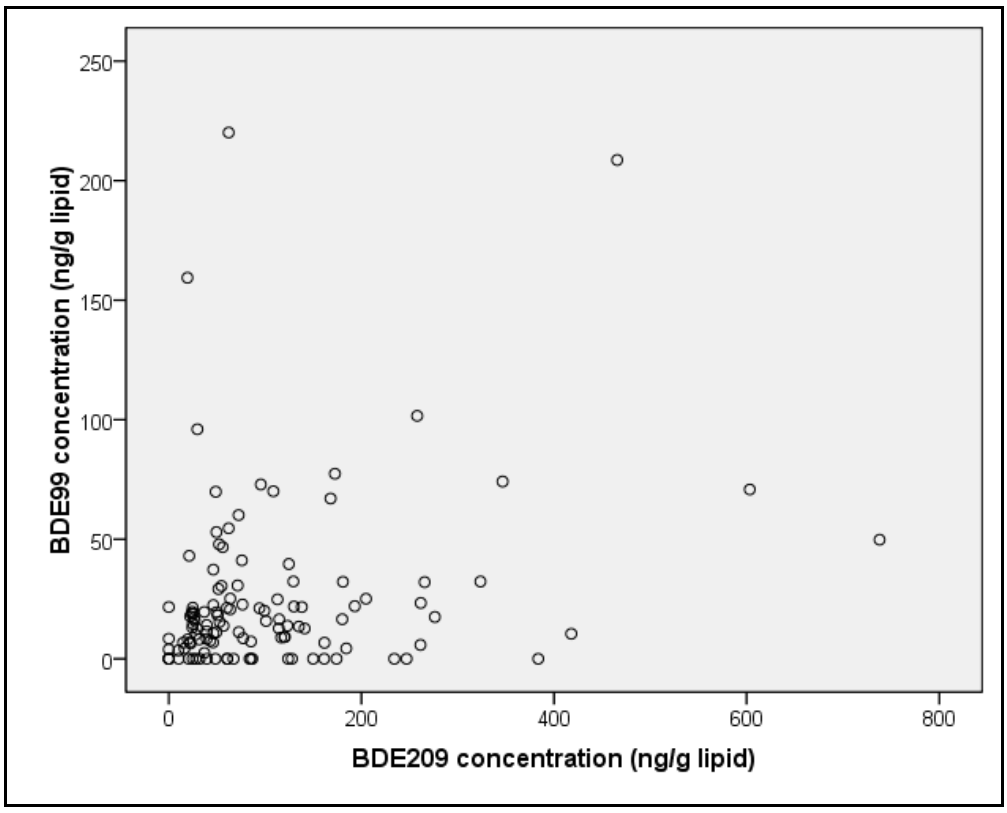


Figure 6.3 An example of poor congener correlation between the relative concentrations of BDE-99 and BDE-209 in otter liver samples.

A principal component analysis (PCA) analyses the correlation between several variables, in this case different congeners. The method is similar to the matrix of correlation coefficients as it allows an assessment to be made of the correlation between concentrations of congeners. Correlation coefficients state that congener X is correlated with congener Y and congener Y is correlated with congener Z, etc. A PCA creates a statistical entity, known as a principal component, which explains the correlation between a group of congeners. The individual congeners are assigned a value, known as a loading, which quantifies the variation in concentrations of that congener as explained by the principal component. The higher the loading, the more accurately the principal component explains the variation in concentrations.

A PCA was performed (using the varimax rotation method) to investigate possible associations between the different BDE congeners (Figure 6.4). The PCA produced six principal components (PCs) with eigenvalues greater than one (the size of the eigenvalue indicates the importance of that component in explaining the total variance in the data). The first two components, accounting for 41.6% and 17.6% of the total variance in the data, respectively, most accurately identify the relationships between congeners (Figure 6.4). The first principal component (PC1) relates to the correlation between congeners BDE-28, -85, -99, -100, -128, -153, -154, -190 and -197. These congeners are generally associated with pent- and octa-BDE commercial mixtures. The correlation between them suggests that these congeners were released into the environment from a similar source. BDE-47 and BDE-66 have relatively high loadings for PC1, but not as high as the other congeners in this group, suggesting that there are likely to be secondary sources for these two congeners.

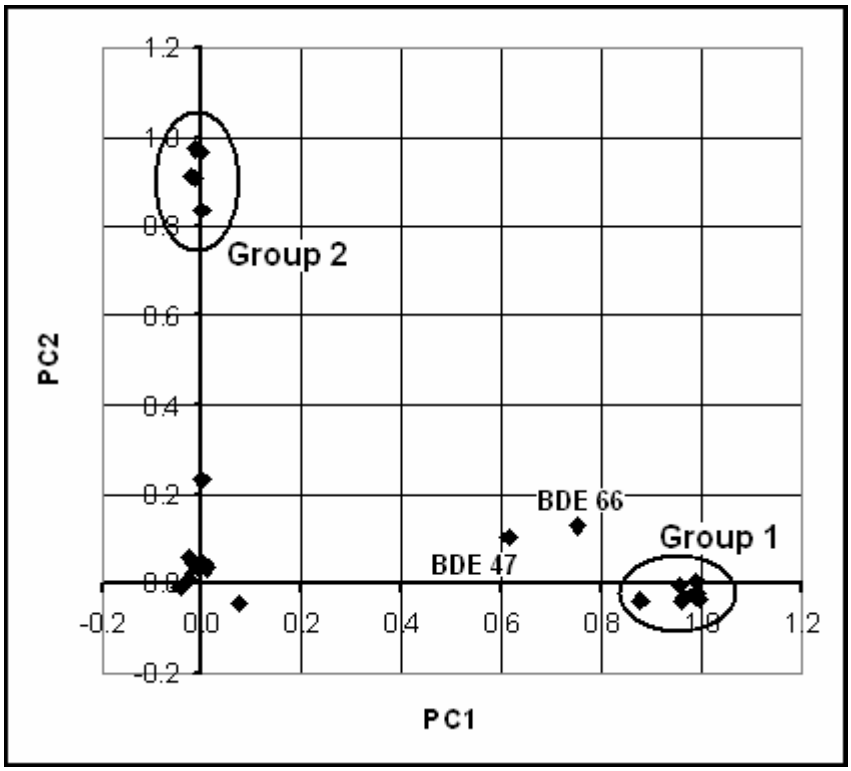


Figure 6.4 Plot of correlation loadings from principal component analysis showing correlations between PBDE congener concentrations in otters.

The second principal component (PC2) shows a significant correlation between the concentrations of BDEs with greater numbers of bromine molecules surrounding the central diphenyl ether structure; BDE-196, -206, -207, -208 and -209 all show strong loadings for this component (Figure 6.4). In this case the common source is likely to be the deca-BDE commercial mixture, as this is the only mixture that contains BDE congeners of this size.

6.2 Potential factors affecting PBDE uptake

BDE congener concentrations detected in individual otter liver samples (Table 6.2) showed considerable variation. Much of this variation came from just a few samples with particularly high concentrations (Figure 6.5). In particular, sample C755 (a sub-adult female collected in 2005 from Dyfed in south Wales (grid ref. SN379488)) contained very high concentrations of many of the lower congeners, including BDE-47, -99, -100 and -153. Sample C657 (an adult female collected in 2003 from Leicestershire, in the midlands (classified as East Anglia for the purposes of this study, grid ref. SP645957)) contained very high concentrations of BDE-47. Interestingly, this level of BDE-47 was not accompanied by significantly high concentrations of other congeners, as might be expected. A third notable case was C743 (an adult male otter collected from a location in Humberside, north east England (grid ref. TA200693) in 2005). The liver sample from this otter contained an especially high concentration of BDE-209, suggesting a high level of exposure to the deca-BDE commercial mixture.

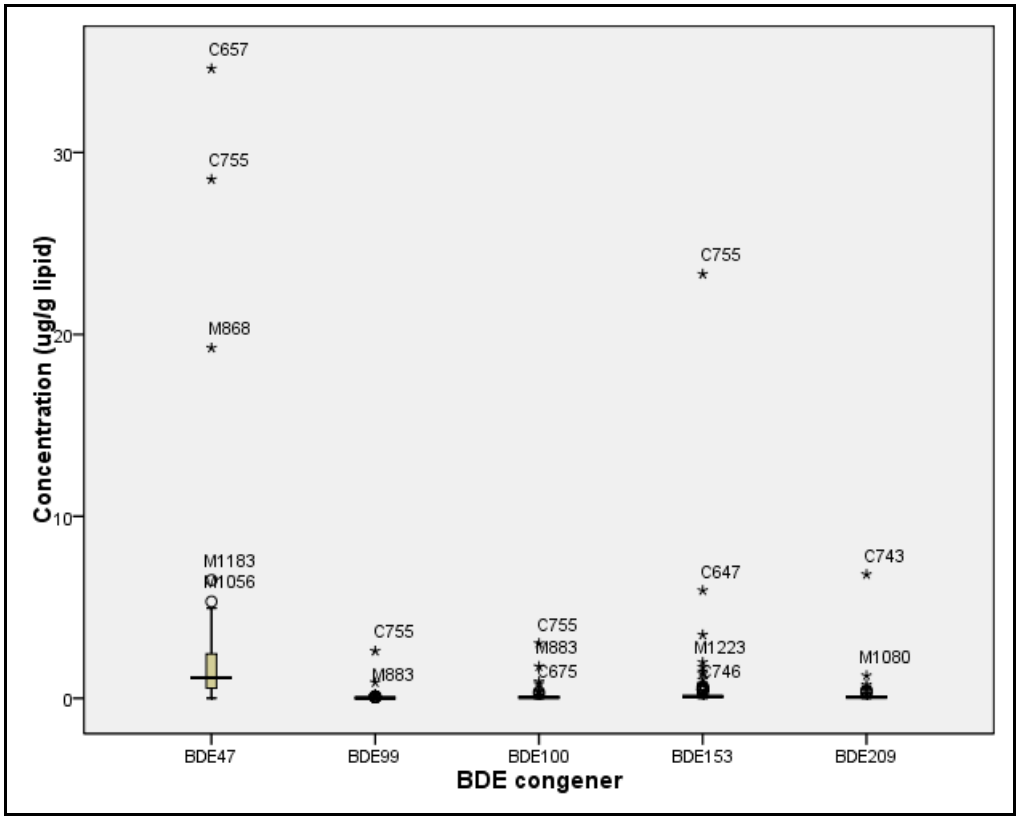


Figure 6.5 Box and whisper plot of concentrations (µg.g^{-1} lipid) for several of the main congeners in the 129 otter liver samples analysed in this study.

The effects of factors such as geographical location, age and body condition, on the PBDE concentrations found in otter liver tissue, were investigated using a multiple regression model. This was to try to identify the causes of the variation in PBDE content observed in otter liver samples. As part of the principal component analysis (introduced in section 6.1.2) principal component scores were assigned to individual otter liver samples, based on the relative concentrations of the congeners correlating with the principal component in question. The scores for the two most influential principal components, PC1 and PC2, were then analysed for their correlation with factors, such as the area of the country they were found or the approximate age of the animal, to try to find the cause of the observed variations in PBDE concentration.

The first factor included in the regression model was geographical location. The samples were split into five areas; northeast England (n = 11), West Midlands (n = 4), East Anglia (n = 13), south Wales (n = 36) and southwest England (n = 64). Otters were grouped in terms of their life stage; adults, sub-adults/juveniles and cubs - using body weight and signs of sexual maturity as a guide (Simpson, 2007; Chadwick, 2007). The gender of the animal was included, as well as the year of the otter's death and whether the animal died as a result of a road traffic accident or from another cause. A final factor was body condition index (*K*) (Kruuk & Conroy, 1996), calculated using the formula:

$$K = W / a L^n$$

Where: W = body weight (kg)

L = total length (m)

a = 5.87 for males and 5.02 for females

n = 2.39 for males and 2.33 for females

The model was first run with all the factors included. Then individual factors that were of the least significance were removed sequentially until the model, and the effects of all factors within it, were significant. The model for PC1 scores, corresponding to the group of BDE congeners with lower numbers of bromine molecules surrounding the central diphenyl ether structure, did not correlate with any of the factors included in the model. Either the statistical model was not powerful enough to detect an effect or none of these factors explain the variation in concentrations of the lower congeners, represented by the PC1 scores. In the regression model of PC2 scores, corresponding to the group of BDE congeners with a higher number of bromine molecules surrounding the central diphenyl ether structure, the only significant factor was geographical area ($t = 2.508$, $df = 117$, $p = 0.014$). This explained 5.1% of the variation ($R^2 = 0.051$). A box and whisper plot of PC2 scores (Figure 6.6) and a plot of mean BDE-209 concentrations (Figure 6.7) showed higher levels of these congeners in northeast England, the West Midlands and East Anglia and lower levels in south Wales and southwest England. It seems that otters in the north and east of England contain, on average, significantly higher concentrations of the higher PBDE congeners than do otters from southwest England and south Wales. This suggests either lower exposure to the deca-BDE commercial mixture in the south west of England and south Wales or that otters in these regions bioaccumulate these congeners to a lesser extent than do otters in more northerly and easterly regions.

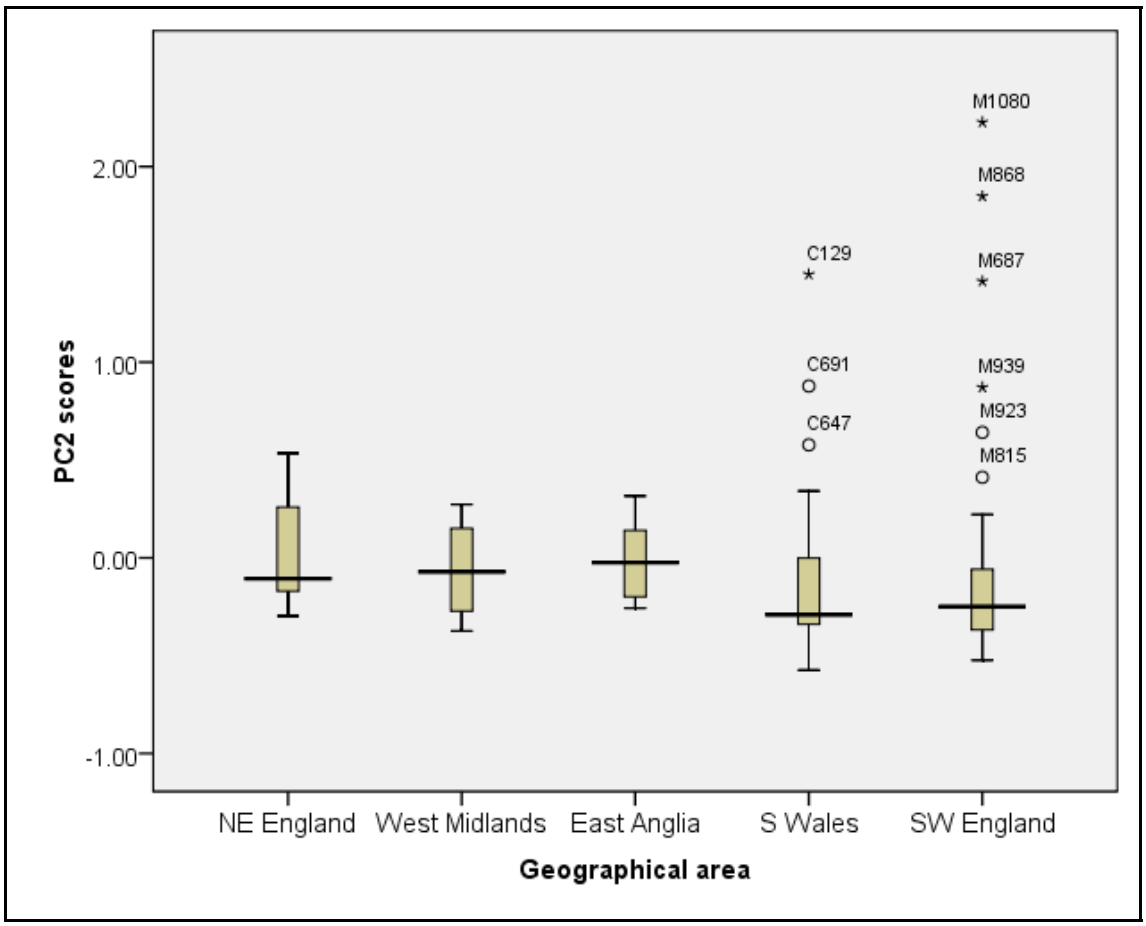


Figure 6.6 Box and whisper plot of PC2 scores against geographical location. One outlying score, not shown on the graph, originates from NE England at a PC2 score of 9.704 (sample C743).

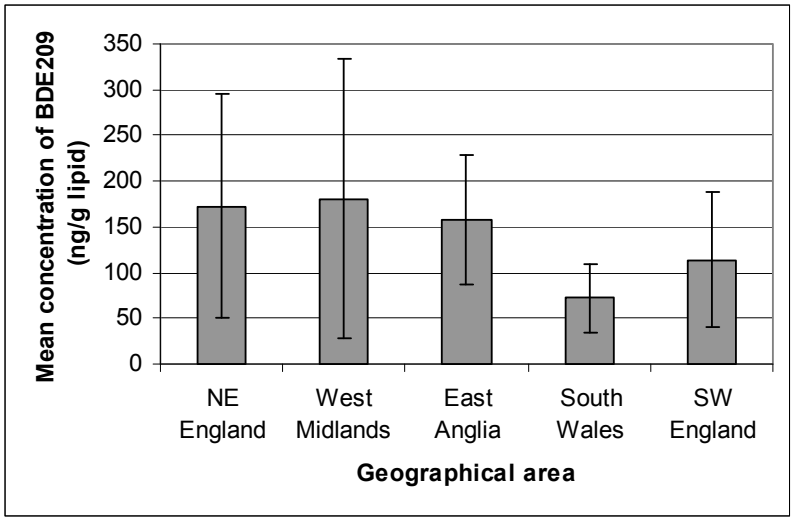


Figure 6.7 Graph of the mean concentrations of BDE209 in the livers of otters from different locations in England and Wales: north east England (n = 11), the West Midlands (n = 4), East Anglia (n = 13), south Wales (n = 36) and south west England (n = 64).

6.3 Comparisons with PCBs and OCs

Several studies have assessed the concentrations of PCBs, DDTs and other organochlorines in the liver tissue of otters collected from across England and Wales (Simpson, 1998, 2007; Chadwick, 2007). It should have been possible to compare PCB and OC concentrations from these previous studies with the PBDE concentrations obtained in this study. However, differences in the laboratory protocols for extraction and chemical analysis might have biased the results of any comparisons. A more accurate comparison would be obtained by analysing the sample extracts from this study, not only for PBDEs, but also a range of PCBs, DDTs and HCB.

Table 6.4 Comparative concentrations of several contaminant groups in otter liver samples.

	Sample nos.	Nos. of congeners	Mean ($ng.g^{-1}$ lipid wt)	Range ($ng.g^{-1}$ lipid wt)
∑PCB	86	25	12928.2	1.8 – 141761.1
∑DDT	86	4	3859.0	L.D. – 46922.5
∑BDE	129	26	3241.9	12.2 – 69882.5
HCB	86	1	260.5	L.D. – 1557.8

L.D. = below the limit of detection

The concentration of ∑PCBs was approximately four fold higher than that of ∑BDE (Table 6.4), despite the similar numbers of congeners analysed, demonstrating that PCBs are still the most dominant of these organic pollutants in otters. As the concentrations of ∑BDEs were similar to those of ∑DDTs, this suggests that PBDEs have become a significant contaminant in wild otter populations in England and Wales.

The most concentrated PBDE congener, BDE-47, was the fourth most concentrated congener of any contaminant analysed in this study (Table 6.5). The mean BDE-47 concentration was approximately half that of the most concentrated PCB congener, PCB-138, and approximately two thirds that of *p,p´*-DDE (Table 6.5). Mean concentrations of BDE-153 and BDE-209 were also significant when compared to the mean concentrations of the most concentrated PCB congeners and DDT breakdown products (Table 6.5).

Table 6.5 Otter liver concentrations of the most prevalent contaminant congeners in animals from England and Wales (n = 86 for PCBs and DDTs, and 129 for PBDE congeners).

Congener [a]	Concentration in $\mu g.g^{-1}$ lipid [b]		
	Mean	Median	Range [c]
PCB-138	3.922	1.955	37.982
pp-DDE	2.990	1.455	33.650
PCB-153	2.753	1.409	37.043
BDE-47	2.591	1.161	34.611
PCB-180	1.782	0.673	24.098
PCB-170	1.301	0.534	13.876
PCB-187	1.043	0.587	10.353
PCB-118	0.548	0.299	9.413
BDE-153	0.526	0.090	23.317
pp-DDD	0.494	0.229	11.049
PCB-194	0.364	0.117	5.258
PCB-203	0.334	0.158	4.359
HCB	0.262	0.199	1.558
PCB-156	0.252	0.117	4.516
PCB-99	0.201	0.116	2.308
BDE-209	0.199	0.064	6.808

[a] in descending order of mean concentration

[b] 1 $\mu g.g^{-1}$ lipid being equal to 1000 $ng.g^{-1}$ lipid

[c] from below limit of detection to the upper limit shown

6.3.1 Correlation of PBDEs with PCBs and OCs

In a PCA comparing the variance in concentrations of all the PCB, OC and PBDE congeners analysed, there was little correlation between congeners from the different compound groups. The first five principal components, explaining a total of 67.3% of the variance, constituted the five main congener groupings (Table 6.6). For PCBs, sixteen of the higher congeners (those with large numbers of chlorines surrounding the central biphenyl structure) corresponded with PC1 (principal component 1), along with the organochlorine hexachlorobenzene (HCB). A group of five of the lower PCB congeners were assigned to PC4. The congener PCB-87 was found to lie between these two groups, with a PC1 loading of 0.722 and a PC4 loading of 0.472. This suggests PCB-87 is partially correlated with both groups of PCB congeners and may originate from two or more sources.

In this analysis the PBDE congeners split in a similar manner to that seen in the PCA analysis of PBDE congeners alone (described in section 6.1.2 *Congener correlations)*. There is a correlation between a suite of 12 BDE congeners, containing lower numbers of bromine molecules, described by PC2. The nona- and deca-BDE congeners, along with BDE-196, are assigned to PC3. Note that PCB-151 partially correlates with these higher congeners, with a PC3 loading of 0.592. The final principal component, PC5, corresponds with DDT and its breakdown products. Interestingly, BDE-35 also aligns with this group. Concentrations of this congener were generally low (mean 1.011 $ng.g^{-1}$ lipid wt, range <0.1 – 30.0 $ng.g^{-1}$ lipid wt) and so the relevance of this finding is not clear.

Table 6.6 The principal components assigned during a PCA analysis of PCB, OC and PBDE congeners combined.

PC [a]	eigenvalues	% of variance explained	cumulative %	corresponding congeners (those with values over 0.5 for the corresponding principal component)
1	14.778	26.869	26.869	PCB-87 [b], -99, -105, -118, -138, -153, -156, -157, -158, -167, -170, -180, -183, -187, -189, -194, -203, HCB
2	10.484	19.062	45.931	BDE-28, -47, -66, -85, -99, -100, -128, -138, -153, -154, -190, -197
3	5.006	9.102	55.033	BDE-196, -206, -207, -208, -209, PCB-151
4	4.217	7.667	62.700	PCB-28, -49, -52, -74, -110
5	2.553	4.642	67.342	*pp*-DDD, *pp*-DDE, *pp*-DDT, BDE-35

[a] Principal component

[b] not exclusively assigned to this principal component

6.4 Discussion - PBDE concentrations in otters

The PBDE congener profile of otters is dominated by BDE-47. As a top predator, otters are likely to bioaccumulate contaminants such as PBDEs as they biomagnify up the food chain (Gama *et al.*, 2006; Whittle *et al.*, 2007). The BDE-47 congener has been shown to dominate the PBDE congener profile of many fish species (Covaci *et al.*, 2004; Schlabach *et al.*, 2004; Hartmann *et al.*, 2007), as well as those of many marine mammals and fish eating birds (Boon *et al.*, 2002; Vorkamp *et al.*, 2004; Braune *et al.*, 2007) indicating that this congener is especially bio-available to many aquatic species. As fish make up a large proportion of the otters diet it follows that, as with other aquatic predators, they will accumulate high concentrations of the lower congeners, in particular BDE-47.

The otter samples analysed in this study contained ΣBDE concentrations approximately equal to those seen in many marine mammal species. For example, in harbour porpoise (*Phocoena phocoena*) from British Columbia ΣBDE ranged from 350 to 2,300 ng.g^{-1} lipid wt (Ikonomou *et al.*, 2002), in southern sea otters (*Enhydra lutris nereis*) from California ΣBDE ranged from 10 to 26,000 ng.g^{-1} lipid wt (mean = 2,200 ng.g^{-1} lipid wt) (Kannan *et al.*, 2007) and in Californian sea lions (*Zalophus californianus*) ΣBDE ranged from 570 to 24,240 ng.g^{-1} lipid wt (Stapleton *et al.*, 2006). It is difficult to draw direct comparisons across these data sets as most marine mammal studies have measured PBDE concentrations in the blubber tissue rather than in the liver. However, concentrations in the otter liver tissue appear to be similar, if not higher, with a ΣBDE range of 12 to 69,883 ng.g^{-1} lipid wt.

The congeners BDE-153 and BDE-209 were found in relatively high concentrations in the otters, paralleling findings from other terrestrial species. The PBDE congener profiles seen in many terrestrial birds species are dominated by the congeners BDE-99

and -153 (de Wit *et al.*, 2006), while in some species particularly high concentrations of BDE-209 have been found (Law *et al.*, 2003; Jaspers *et al.*, 2006). In the few studies that have investigated PBDE concentrations in terrestrial mammals, BDE-153 and -209 have been found in relatively high concentrations. In contrast, the higher congeners, including BDE-209, are rarely observed in marine biota and when they are it is usually only in trace amounts (Boon *et al.*, 2002; de Boer *et al.*, 2003).

The otter liver tissue analysed in this study contained particularly high concentrations of the congener BDE-209, with a range of <8.5 - 6,808 $ng.g^{-1}$ lipid wt, mean of 167 $ng.g^{-1}$ lipid wt and median concentration of 62.2 $ng.g^{-1}$ lipid wt. Some of the highest BDE-209 concentrations seen in birds have been recorded in the Chinese pond heron (*Ardeola bacchus*, with a range of 3.1 - 290 $ng.g^{-1}$ lipid wt (Lam *et al.*, 2007)), and in Glaucous gulls (*Larus hyperboreus*, with a range of 23 - 53 $ng.g^{-1}$ lipid wt (Herzke *et al.*, 2003)). Voorspoels *et al.* (2006) found BDE-209 concentrations in red foxes (*Vulpes vulpes*) from Belgium ranging from <9.1 - 760 $ng.g^{-1}$ lipid wt (median of <9.1 $ng.g^{-1}$ lipid wt), with BDE-209 making up to over 70% of the PBDE congener profile in some of the animals. In racoon dogs (*Nyctereutes procyonoides*) in Japan, BDE-209 was the dominant congener in a ΣBDE ranging from 0.36 to 250 $ng.g^{-1}$ lipid wt (Kunisue *et al.*, 2008). It would seem that although BDE-209 concentrations observed in the otters are some of the highest observed in biota, they are not wildly different from those seen in some other top predatory species.

The high concentrations of both the lower PBDE congeners, in particular BDE-47, and the higher PBDE congeners, such as BDE-153 and BDE-209, in liver tissue of otters, suggests these semi-aquatic mammals take up BDEs from both the aquatic and the terrestrial environments. This is supported by the separation of the higher congeners and lower congeners, when plotted on a PCA. Note that the BDE-153 congener is an exception, it separates out with the lower congeners, despite its apparent link with accumulation from the terrestrial environment. The fact that BDE-47 does not correlate completely with the suite of other lower congeners is probably as a consequence of diet, fish species tend to accumulate disproportionately high concentrations of this particular congener (Covaci *et al.*, 2004; Schlabach *et al.*, 2004; Hartmann *et al.*, 2007).

The concentrations of PBDEs in otter liver tissue are not that dissimilar to concentrations observed for other organic and persistent contaminants; for example, concentrations of BDE-47 are similar to those of some of the most concentrated PCB congeners and DDT breakdown products. PBDEs can behave in a similar fashion to PCBs in terms of their endocrine disrupting and neurological effects. Despite their lower toxicological potency, compared to some of the PCB congeners, the concentrations of PBDE congeners found in the liver tissue of otters are sufficient to give rise to concerns over potential negative health effects (Zhou *et al.*, 2001; Hallgren *et al.*, 2001; Viberg *et al.*, 2003; Branchi *et al.*, 2005).

7 Conclusions

Microsatellite genotyping of spraint samples:

- Analysis of spraint samples collected from the River Camel in Cornwall revealed that a minimum of 16 otters used the river system at some point over the two seasons of study.
- Linking spraint microsatellite genotypes to information regarding the date and location of sample collection enabled a detailed picture of the range and movement of otters to be constructed.
- The increased number of microsatellite loci used for genotype analysis of otter spraint samples in this study allowed groups of related individuals to be inferred, based on genetic relatedness.
- Analysis of genotyping success rates showed that anal jelly samples were significantly more likely to produce a genotype than spraint samples and samples collected in cold (snowy or frosty) weather were significantly more likely to produce a genotype than those collected under warmer weather conditions.

The River Itchen otter population:

- Analysis of population differentiation (F_{ST} analysis), based on microsatellite genotypes, indicated that the River Itchen population and an otter population from Dorset are genetically more similar to each other than either of them are to the Cornish otter population.
- This finding is supported by an allele-sharing analysis which placed Itchen and Dorset otters together and away from Cornish otters in a neighbour-joining tree.
- Microsatellite genotyping analysis of otter tissue samples revealed that the River Itchen and Dorset otter populations are more genetically diverse than the Cornish population, both in terms of allelic richness and heterozygosity. In the case of the Itchen population, given its demographic history, it may be that a significant proportion of this population's genetic diversity is due to the introduction of novel genetic material from the release of captive bred otters.
- Haplotype analysis revealed the presence of the haplotype Lut3 in two of the nine otters analysed from the Itchen. As this haplotype has been found in a high proportion of captive bred otters and has not been found in any 'natural' UK populations, this can be interpreted as evidence that released captive bred otters did interbreed with Itchen otters and that descendants of these otters are present in the extant River Itchen population.

PBDE concentrations in otter liver tissue:

- The total (Σ)BDE concentrations measured in otter liver samples are similar to those measured in the blubber tissue of many marine mammal species, indicating that, as a top semi-aquatic predator, otters are accumulating high concentrations of this pollutant.
- The PBDE congener profile found in otter tissues indicates take up from both the aquatic (congeners with low numbers of bromine molecules) and terrestrial (relatively high concentrations of BDE-153 and BDE-209) environments.
- Correlations between the concentrations of the different BDE congeners found in liver tissue reflect the profiles of commercial PBDE mixtures, suggesting that these may be the original sources of contamination.
- Concentrations of the suite of congeners associated with the Deca-BDE commercial mixture, i.e. BDE-206, -207, -208 and -209, were significantly correlated with the region the samples originated from. Otters from the north and east of England contained, on average, significantly higher concentrations of these congeners than those in south Wales or southwest England.
- The concentrations of BDE-209 measured in otter liver samples are some of the highest, if not the highest, concentrations measured in any species of animal to date.
- Concentrations of ΣBDEs in otter liver tissue are similar to those of ΣDDTs and are approximately a quarter those of PCB concentrations, with mean ΣBDE concentrations of 3,242 $ng.g^{-1}$ lipid wt, mean ΣDDT concentrations of 3,859 $ng.g^{-1}$ lipid wt and mean ΣPCB concentrations of 12,928 $ng.g^{-1}$ lipid wt.

8 Future Research

Use of the spraint genotyping technique:

- The panel of eleven loci used in this study are now well optimised for future work. The development of more microsatellites for genotype analysis in otters is to be welcomed, but we suggest that the methodology is now suitably developed. Research should now focus on using these markers to conduct in depth population genetic studies in the light of the data on otter habitat use, home range boundaries, and migration ranges/patterns.
- In particular, longer term spraint genotyping studies, carried out on the same river system over a number of years, would add to our knowledge of otter behaviour and survival rates. This information could be particularly valuable in supporting and validating the monitoring of otters by direct observation approaches.
- Previous studies, and to some extent this project, have been hampered by small sample sizes. This precludes the use of the powerful software packages now used routinely in other disciplines, e.g. fisheries, to investigate such issues as population subdivision, temporal stability of populations and assignment of lone individuals to a baseline population. We suggest that the strength of the findings from future work will be increased significantly by focusing – where possible – on the collection and analysis of larger numbers of samples.
- The spraint genotyping technique readily lends itself to assessing the impact of specific human activities (e.g. road traffic accidents (RTA)) on otter populations. Information on the age, sex and breeding status of RTA animals can provide significant insight into the likely implications for a resident otter population; for example, the death of a breeding female is likely to have a far greater impact on a population that the death of a juvenile male. The use of the spraint genotyping technique to identify the boundaries of otter home ranges would be of use in predicting and assessing the effectiveness of conservation/mitigation techniques, i.e. for redirecting habitat use away from an area of potential conflict.

Assessing the impact of the release of captive-bred otters:

- A more in depth microsatellite genotyping project, using greater numbers of otter tissue samples from areas across England and Wales, would help to assess the extent to which the release of captive-bred otters has altered the genetic composition of the population.
- Investigations into the geographical origins of the otters used to found the Otter Trust captive breeding programme would help in assessing the genetic integrity of the resultant populations and the potential for outbreeding to have deleterious effects in the offspring of released and wild otter matings. For example, in the River Itchen, the geographic origins of the founding otters requires investigation by comparing the mitochondrial

haplotypes of extant Itchen otters with animals from the region(s) identified as the origins of otters used in the captive breeding programme.

Concentrations of PBDEs in otter liver tissue:

- The PBDE concentrations found in the liver tissue of otters from England and Wales was comparable to concentrations observed in many marine top predators. The concentrations of PBDEs in otter liver tissue are comparable with concentrations of some other key organic pollutants, the PCBs. Given this finding, the continued monitoring of PBDE concentrations in the liver tissue of otters from England and Wales would be advisable.
- High concentrations of nona- and deca-BDE congeners were found in the otters analysed in this study. The continued monitoring of these congeners would be beneficial in helping to develop our understanding of the potential negative effects they may have on otter populations.
- It is important to understand how otters accumulate the higher congeners (e.g. BDE-153 and BDE-209), which are associated more with the terrestrial than the aquatic environment and do not appear to be linked to uptake via the diet (Voorspoels *et al.*, 2006).
- Investigations into possible links between PBDE concentrations and negative health effects are much needed so that we understand whether the concentrations observed in English and Welsh otter populations are likely to cause significant adverse health effects. The focus should be on assessments of thyroid function and potential reproductive and neurological damage resulting from PBDE exposure.
- As a possible surrogate/support for studies on PBDE effects in otters, wild mink could be used. This species is more easily accessible in terms of sample collection and occupies a similar ecological niche. Mink have been used previously, and with good success, to assess the potential toxicological effect of other organic pollutants in otters (Jensen *et al.*, 1997; Bächlin & Bergman, 1992; Kihlström *et al.*, 1992).

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Glossary

Allele	One of a series of different forms of a gene. As a diploid organism, otters will possess one or two alleles at each locus/microsatellite marker (see also Microsatellite allele).
Anal jelly	A gelatinous anal-gland secretion that otters deposit in addition to digested feacal matter (see Spraint).
Base pairs (bp)	The building blocks of DNA, quoted as pairs due to the linkage of pairs of bases, one on each strand of the two strands of DNA making up the double helix structure.
BDE congeners	These are the different molecular structures formed by the variations in numbers and locations of bromine molecules surrounding the central diphenyl ether structure.
DCM	The solvent dichloromethane.
DDTs	Dichloro diphenyl trichloroethanes were extensively used, both for pest control and as a crop insecticide, until their potential human and environmental impacts were recognised and use of the substance was banned.
Dieldrin	This chlorinated hydrocarbon, based on the active ingredient hexachlorocyclopentadiene, was used as an insecticide in Britain in the 1950s to early 1970s.
Genotype	The genetic composition of an individual, usually in reference to the specific genetic markers being studied (see also Microsatellite genotype)
HCBs	Hexachlorobenzene is a fungicide, formerly used in the treatment of cereal crops.
Higher BDEs	The set of BDE congeners containing larger numbers of bromines, generally between eight and ten, around the central diphenyl ether structure.
IMS	Industrial methylated spirits are derived from ethanol, with methanol used as an additive to render the resulting solvent unsafe for human consumption.
Locus (plural loci)	Refers to a fixed position in the genome (see also Microsatellite locus).
Lower BDEs	The set of BDE congeners containing fewer, generally up to seven or eight, bromines around the central diphenyl ether structure.
Microsatellite allele	Mutational events create different sizes, or versions, of microsatellite alleles, which can then become fixed in a population.
Microsatellite genotype	The specific allelic make up of an individual for the suite of microsatellite loci used in a particular study.
Microsatellite locus	Refers to a microsatellite found at a specific location within the genome of the species being studied.

Microsatellites	Stretches of DNA consisting of short sequence repeats. For example, $CA_{(n)}$ would be a repeat, repeated *n* times in a row, of the short sequence CA.
Mitochondria	The organelle, or subunit of a cell involved in the generation of ATP, the cells store of 'useable' energy.
Mitochondrial control region	A non-coding section of mitochondrial DNA, responsible in part for the regulation of replication and expression of mitochondrial DNA and genes. Also known as the 'D-loop'.
Mitochondrial DNA	A circular DNA molecule held within the mitochondria, and separate from DNA contained within the nucleus of a cell (see Nuclear DNA).
Mitochondrial haplotype	The identity given to a particular mitochondrial gene DNA sequence. Variation in haplotype identity indicates variation in the DNA sequence of the region of mitochondrial DNA being studied.
Nuclear DNA	This body of DNA, contained within the cell's nucleus, contains the vast majority of a cell's genetic material. Every nucleated cell contains a complete copy of the genome inside the nucleus (but see also mitochondrial DNA).
OCs	Organochlorines, including compounds such as DDTs, dieldrin and HBCs.
PCBs	Polychlorinated biphenyls.
PBDEs	Polybrominated diphenyl ethers.
Spraint	Otter faecal matter.

Appendix 1 Spraint sample database set-up

The data produced from the spraint genotyping project was stored in an Access database. The following four tables summarise the data format used.

Table A1.1. Sample Data.

Field name	Data type	Brief description
Lab ID	Text	The laboratory sample identification code
Tube number	Text	Assigned by collector in the field
Sample type	Combo box	Spraint, jelly or spraint and jelly mixed
Site code	Combo box	Designated reference code for collection site recognition
Sampling year	Combo box	Identification of the season of collection
Date of collection	Date/Time	Date of sample collection
Time of collection	Date/Time	Precise time of sample collection recorded by collector
Collector	Combo box	Identification of the person responsible for the collection of the sample
Weather conditions	Combo box	Interpreted using information provided by the sample collector
Substrate under sample	Combo box	Interpreted using information provided by the sample collector
Degree of exposure	Combo box	Interpreted using information provided by the sample collector
Extracted	Yes/No	DNA extraction has been attempted
Date extracted	Date/Time	Date of the first DNA extraction attempt
Genotyped	Yes/No	Sample extract shows signs of having produced DNA
Full genotype acquired	Yes/No	A full usable genotype has been obtained from the sample
CEQ dataset	Text	Information regarding the storage of data from fragment analysis runs
Comments	Memo	Comments relating to this sample - the collection or analysis thereof
Spraint description	Combo box	Brief description of spraint material
Spraint size	Combo box	Amount of material collected
IMS colouration	Combo box	Degree of colouration observed in the lab
Spraint to IMS ratio	Combo box	Brief description of ratio observed

Table A1.2. Sample Site.

Field name	Data type	Brief description
Site code	Text	Designated reference code for collection site recognition
Site name	Text	Name tag to aid recognition of the site
Grid reference	Text	OS grid reference
Watercourse	Text	Tributary name, etc.

Table A1.3. Lab Data: Genotypes.

Field name	Data type	Brief description
Lab ID	Text	The laboratory sample identification code
Primer	Combo box	Microsatellite locus ID
Allele 1	Text	First allele size
Allele 2	Text	Second allele size, or duplicate of first
Sequencer run	Text	The data output file in which the information is contained
Sequencer well	Text	The sequencer well in which the PCR product was run
PCR	Text	The PCR run from which the product was obtained
PCR well	Text	The well in which the sample was loaded in the PCR
Data entry number	AutoNumber	Automatically assigned identification number - for basis of running this database only

Table A1.4. Lab Data: Microsatellite Alleles.

Field name	Data type	Brief description
Lab ID	Text	The laboratory sample identification code
Otter Number	Text	Assignment of otter identity
Genotype quality	Combo box	Usefulness of the data produced, eg if the sample has been successfully genotyped at very few microsatellite loci then the quality of the overall genotype is poor
Lut435 allele1	Combo box	Allele size
Lut435 allele2	Combo box	Allele size
Lut701b allele1	Combo box	Allele size
Lut701b allele2	Combo box	Allele size
Lut717b allele1	Combo box	Allele size
Lut717b allele2	Combo box	Allele size

Appendix 2 Spraint genotyping results

A2.1 River Itchen genotypes

Lab ID	Otter	Lut717b		Lut435		04OT14		04OT17		Lut832		Lut733		Lut782		Mvis075	
ITC013	A	04	04	01	01	01	01	03	03	04	04	01	02	02	02	02	02
ITC016	B	04	04	01	01	01	01	03	03	01	02	02	02	01	02	01	02
ITC023	C	03	04	01	04	01	02	02	03	04	04	01	01	02	02	01	02
ITC020	D	03	04	01	04	01	02	02	03	--	--	01	03	01	02	01	02

A2.2 River Camel genotypes

Lab ID	Otter	Lut717b		04OT17		Lut435		04OT14		RIO11		Lut701b		Lut832		04OT04b		04OT07		04OT19b		RIO18	
CAM141	A	01	01	01	02	01	03	03	03	02	03	03	03	03	05	02	02	01	02	02	02	02	02
CAM302	A	01	01	01	02	01	03	03	03	02	03	03	03	03	05	02	02	01	02	02	02	02	02
CAM344	A*	01	01	01	02	01	03	03	04	02	03	03	03	03	05	02	02	--	--	02	02	02	02
CAM035	B	01	01	01	01	03	03	03	03	02	02	04	04	04	04	01	02	06	06	01	01	02	02
CAM155	B	01	01	01	01	03	03	03	03	02	02	04	04	04	04	01	02	--	--	01	01	02	02
CAM160	B	01	01	01	01	03	03	03	03	02	02	--	--	--	--	--	--	--	--	--	--	--	--
CAM489	C	01	03	01	02	03	03	03	03	02	03	03	04	04	04	01	01	02	06	01	02	02	02
CAM007	C	01	03	01	02	03	03	03	03	--	--	--	--	--	--	--	--	--	--	--	--	--	--
CAM132	D	01	03	01	02	01	03	03	03	02	02	04	04	03	04	01	02	02	06	02	02	02	02
CAM356	E	01	03	01	02	01	03	03	03	02	03	03	04	04	05	01	02	01	02	02	02	02	02
CAM004	E	01	03	01	02	01	03	03	03	02	03	03	04	04	05	01	02	01	02	02	02	02	02
CAM170	E	01	03	01	02	01	03	03	03	02	03	03	04	04	05	01	02	01	02	02	02	02	02
CAM495	E	01	03	01	02	01	03	03	03	02	03	03	04	04	05	01	02	01	02	02	02	02	02
CAM491	E	01	03	01	02	01	03	03	03	02	03	03	04	04	05	01	02	01	02	02	02	02	02
CAM013	E	01	03	01	02	01	03	03	03	02	03	03	04	04	05	01	02	01	02	02	02	02	02
CAM012	E	01	03	01	02	01	03	03	03	02	03	03	04	04	?	--	--	01	01	02	02	02	02
CAM062	E	01	03	01	02	01	03	03	03	02	03	03	04	04	05	01	02	01	02	02	02	02	02
CAM265	F	01	03	01	01	01	01	03	03	02	02	03	03	03	05	02	02	01	03	02	02	02	02
CAM290	F	01	03	01	01	01	01	03	03	02	02	03	03	03	05	02	02	01	03	02	02	02	02
CAM200	F	01	03	01	01	01	01	03	03	02	02	03	03	03	05	02	02	01	03	02	02	02	02
CAM271	F	01	03	01	01	01	01	03	03	02	02	03	03	03	05	02	02	01	03	02	02	02	02
CAM222	F	01	03	01	01	01	01	03	03	02	02	03	03	03	05	--	--	01	03	02	02	02	02
CAM260	F	01	03	01	01	01	01	03	03	02	02	03	03	03	05	02	02	01	03	--	--	02	02

CAM080	G	03	03	01	01	03	03	03	04	02	03	04	04	04	05	01	01	01	01	02	02	02	02
CAM074	G	03	03	01	01	03	03	03	04	--	--	--	--	04	05	--	--	--	--	--	--	--	--
CAM215	G	03	03	01	01	03	03	03	04	--	--	--	--	--	--	--	--	--	--	--	--	--	--
CAM142	G	03	03	01	01	03	03	03	04	--	--	--	--	--	--	--	--	--	--	--	--	--	--
CAM192	G	03	03	01	01	03	03	03	04	--	--	--	--	--	--	--	--	--	--	--	--	--	--
CAM156	H	03	03	01	01	03	03	03	03	02	03	04	04	03	04	02	02	02	06	02	02	02	02
CAM257	I	03	03	01	01	01	01	03	03	02	02	03	03	03	04	--	--	02	03	01	02	--	--
CAM084	I	03	03	01	01	01	01	03	03	02	02	--	--	--	--	--	--	--	--	01	02	--	--
CAM258	J	01	03	01	01	01	03	03	03	02	02	03	03	04	04	--	--	02	02	02	02	02	02
CAM263	K	01	03	01	01	01	03	03	03	02	02	03	03	03	03	--	--	01	02	--	--	02	02
CAM304	L	03	03	01	01	03	03	03	03	02	02	04	04	04	04	02	02	02	06	02	02	02	02
CAM303	L	03	03	01	01	03	03	03	03	02	02	04	?	04	04	02	02	02	06	02	02	02	02
CAM120	L?	03	03	01	01	03	03	03	03	--	--	--	--	04	04	--	--	--	--	--	--	--	--
CAM485	M	01	01	01	01	01	01	03	03	02	02	03	04	03	03	02	02	02	03	02	02	02	02
CAM469	M	01	01	01	01	01	01	03	03	02	02	03	04	03	05	02	02	02	03	02	02	02	02
CAM454	N	01	01	02	02	03	03	03	03	02	02	04	04	04	05	01	02	02	03	02	02	02	02
CAM445	O	01	03	01	01	03	03	03	03	02	02	03	03	04	04	--	--	--	--	01	01	02	02
CAM427	P	03	03	01	01	01	03	03	03	02	03	03	04	04	04	01	02	01	02	02	02	02	02
CAM002	C?	01	03	01	02	03	03	03	03	--	--	--	--	--	--	--	--	--	--	--	--	--	--
CAM054	C?	01	03	01	02	03	03	03	03	--	--	--	--	04	04	--	--	--	--	--	--	--	--
CAM145	A?	01	01	01	02	01	03	03	03	--	--	--	--	--	--	--	--	--	--	--	--	--	--

A2.3 River Camel spraint sample field data

Lab ID	Otter	Tube no.	Date	Time	Collection site reference	Collection site	Grid reference
CAM141	A		06 Feb 2006		CCEN005	Treglyn Farm	SW976765
CAM302	A	001	26 Nov 2006	11:00	CCEN005	Treglyn Farm	SW976765
CAM344	A*	001	29 Oct 2006	08:15	CCES002	Toll Dowrgi	SW972738
CAM035	B	009	30 Jan 2006	08:30	CC002	Tregoodwell	SX115837
CAM155	B	024	25 Feb 2006	11:05	CC004A	Trecarne Confluence	SX097805
CAM160	B	001	28 Dec 2005	08:15	CC001	Slaughter Bridge	SX109855
CAM489	C	020	06 Dec 2005	10:00	Butterwell	Butterwell	SX021676
CAM007	C	118	29 Jan 2007		Butterwell	Butterwell	SX021676
CAM132	D		27 Nov 2005		CC015 A2	Bishops Wood	SX011701
CAM356	E	103	03 Nov 2006		Butterwell	Butterwell	SX021676
CAM004	E	023	08 Dec 2005	10:00	Butterwell	Butterwell	SX021676
CAM170	E	012	27 Feb 2006	10:30	CC015 E4	Grogley Gauging station	SX017680
CAM495	E	115	01 Jan 2007		Butterwell	Butterwell	SX021676
CAM491	E	117	20 Jan 2007		Butterwell	Butterwell	SX021676
CAM013	E	021	07 Dec 2005	10:30	Butterwell	Butterwell	SX021676
CAM012	E	015	27 Nov 2005	11:00	Butterwell	Butterwell	SX021676
CAM062	E	031	10 Dec 2005	10:00	Butterwell	Butterwell	SX021676
CAM265	F	002	21 Dec 2006	09:47	CCES006	Dennis Cove	SW921744
CAM290	F	003	14 Jan 2007	19:15	CCES006	Dennis Cove	SW921744
CAM200	F	003	16 Mar 2006	08:00	CCES002	Toll Dowrgi	SW972738
CAM271	F	002	21 Dec 2006	09:05	CCES004	Pinksons Point	SW945736
CAM222	F	002	16 Mar 2006	07:55	CCES002	Toll Dowrgi	SW972738
CAM260	F	001	21 Dec 2006	09:45	CCES006	Dennis Cove	SW921744

CAM080	G	002	29 Nov 2005	09:01	CCES006	Dennis Cove	SW921744
CAM074	G	002	02 Jan 2006	10:05	CCES005	Old Town Point	SW938740
CAM215	G	003	09 Feb 2006	09:00	CCES006	Dennis Cove	SW921744
CAM142	G	004	16 Mar 2006	09:10	CCES005	Old Town Point	SW938740
CAM192	G	001	09 Feb 2006	08:50	CCES006	Dennis Cove	SW921744
CAM156	H	008	04 Feb 2006		CC015 F3	Ruthernbridge Bridge	SX013669
CAM257	I	003	31 Jan 2007	08:55	CCES005	Old Town Point	SW938740
CAM084	I	001	29 Nov 2005	09:00	CCES006	Dennis Cove	SW921744
CAM258	J	001	01 Jan 2007	08:50	CC016	Bridge on Wool	SW991724
CAM263	K	001	15 Feb 2007	07:50	CC016	Bridge on Wool	SW991724
CAM304	L	026	01 Jan 2007		CC015 E2	Camel Ruthern junction	SX017680
CAM303	L	023	28 Oct 2006		CC015 F3	Ruthernbridge Bridge	SX013669
CAM120	L?	005	02 Jan 2006		CC015 E2	Camel Ruthern junction	SX017680
CAM485	M	001	30 May 2007	08:50	CC016Pe	Pendavey Bridge	SX004714
CAM469	M	002	30 May 2007	08:55	CC016Pe	Pendavey Bridge	SX004714
CAM454	N	001	30 Jun 2007	13:50	CDL002	Bradford Bridge	SX120750
CAM445	O	001	29 Jun 2007	20:40	CDL002	Bradford Bridge	SX120750
CAM427	P	031	02 Apr 2007		CC015 E4	Grogley Gauging station	SX017680
CAM002	C?	035	12 Dec 2005	10:00	Butterwell	Butterwell	SX021676
CAM054	C?	037	13 Dec 2005	10:15	Butterwell	Butterwell	SX021676
CAM145	A?	002	07 Mar 2006	07:38	CC005	Gam bridge	SX088778

Appendix 3 Tissue sample data

A3.1 Sample collection data

Ref ID	Year of death	Grid reference	Geographical group for:	
			microsatellite analysis	mitochondrial haplotyping
M60	2001	SX247843	Cornwall	Cornwall
M294	2002	SW942690	Cornwall	Cornwall
M299	2002	SW842615	Cornwall	Cornwall
M315	2002	SX253551	Cornwall	Cornwall
M377	2003	SU352238	River Itchen	River Itchen
M385	2003	SU545328	River Itchen	River Itchen
M386	2003	SU459473	River Itchen	River Itchen
M638	2004	SW817459	Cornwall	
M650	2004	SU576391	River Itchen	River Itchen
M659	2004	SW825452	Cornwall	
M685	2004	TF780160		East Anglia
M730	2004	SU146089	River Itchen	River Itchen
M739	2004	SW777324	Cornwall	
M778	2004	SX222849	Cornwall	Cornwall
M813	2004	SU365117	River Itchen	
M815	2004	SU463414	River Itchen	River Itchen
M868	2005	SX037632	Cornwall	Cornwall
M883	2005	SN185448		South Wales
M887	2005	SX228917	Cornwall	Cornwall
M894	2005	SW945605	Cornwall	Cornwall
M909	2002	TL902693		East Anglia
M923	2005	SW908626	Cornwall	Cornwall
M927	2005	SO081603		South Wales
M984	2005	SX135736	Cornwall	Cornwall
M1005	2005	SX107601	Cornwall	Cornwall
M1033	2005	SY964986	Dorset	River Itchen
M1035	2005	SS296053	Cornwall	
M1041	2005	TL008567		East Anglia
M1053	2005	SY996907	Dorset	
M1056	2005	SZ008991	Dorset	
M1057	2005	SZ008991	Dorset	
M1060	2005	SX186877	Cornwall	Cornwall
M1061	2005	SY917863	Dorset	
M1064	2004	SU351236	River Itchen	River Itchen
M1082	2005	SU140050	Dorset	River Itchen
M1097	2005	TG238078		East Anglia

M1118	2006	TG430152		East Anglia
M1131	2006	TL795120		East Anglia
M1190	2006	TL176617		East Anglia
C593	2003	SN126052		South Wales
C594	2003	SN162070		South Wales
C595	2003	SM959241		South Wales
C612	2003	SO200122		South Wales
C637	2003	SO165357		South Wales
C644	2003	ST525914		South Wales
C645	2003	SO404020		South Wales
C648	2003	SO304205		South Wales
C649	2003	SO412032		South Wales
C691	2004	SN637122		South Wales
C703	2005	SS505985		South Wales
C1016	2008		River Itchen	

Note: Ref IDs beginning with M were processed at the Wildlife Veterinary Investigation Centre. Ref IDs beginning with C were processed at the Cardiff University Otter Project

A3.2 Allele frequency data

Microsatellite locus	Allele	Cornwall (n = 16) *	Dorset (n = 5) *	Itchen (n = 9) *	Total (n = 30) *
Lut701b	01	-------	0.200	-------	0.034
	02	-------	0.300	0.375	0.155
	03	0.563	0.400	0.375	0.483
	04	0.438	0.100	0.250	0.328
Lut717b	01	0.375	0.300	0.056	0.267
	02	0.031	-------	-------	0.017
	03	0.594	0.300	0.389	0.483
	04	-------	0.400	0.556	0.233
04OT14	01	-------	-------	0.333	0.100
	02	-------	-------	0.111	0.033
	03	0.938	1.000	0.444	0.800
	04	0.063	-------	0.111	0.067
04OT17	01	0.906	0.600	0.278	0.667
	02	0.094	0.400	0.444	0.250
	03	-------	-------	0.278	0.083
Lut435	01	0.406	0.500	0.556	0.467
	02	0.063	0.200	-------	0.067
	03	0.531	0.300	0.278	0.417
	04	-------	-------	0.167	0.050
RIO11	01	-------	0.400	0.111	0.100
	02	0.719	0.200	0.722	0.633
	03	0.281	0.400	0.167	0.267
04OT04b	01	0.406	0.600	0.722	0.533
	02	0.594	0.100	-------	0.333
	03	-------	0.100	-------	0.017
	04	-------	0.200	0.278	0.117
04OT19	01	0.313	0.200	-------	0.200
	02	0.688	0.200	0.444	0.533
	03	-------	0.600	0.556	0.267
RIO18	01	0.063	0.100	-------	0.050
	02	0.938	0.900	1.000	0.950
Lut832	01	-------	0.100	0.222	0.086
	02	0.167	0.200	0.278	0.207
	03	0.133	0.100	0.111	0.121
	04	0.400	0.500	0.389	0.414
	05	0.300	0.100	-------	0.172
04OT07	01	0.250	0.100	0.222	0.217
	02	0.188	-------	-------	0.100
	03	0.281	0.200	0.222	0.250
	04	-------	0.100	-------	0.017
	05	-------	0.100	0.333	0.117
	06	0.281	0.500	0.222	0.300

* n = number of individuals genotyped, i.e. number of alleles is twice this value

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