

# Molecular analysis of the trophic interactions of British reptiles

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Thesis submitted for the degree of Doctor of Philosophy,  
Cardiff School of Biosciences,  
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## Thesis Summary

For this Thesis, molecular techniques were developed and applied in the analysis of the diet of British reptiles. Such approaches provide an opportunity to overcome the biases of conventional dietary analysis.

Faeces were collected from smooth snakes (*Coronella austriaca*), grass snakes (*Natrix natrix*) and slow worms (*Anguis fragilis*) each month over two years. DNA was extracted and, through the use of PCR primers, cloning and pyrosequencing, seasonal and ontogenetic changes in their diet was investigated. The results establish, for the first time, that prey DNA is detectable in reptile faeces.

Predation by slow worms on pulmonates and earthworms was much higher than previous studies have suggested, with seasonal patterns in predation but no ontogenetic ones. Predation on *Arion* slugs was higher in females in the spring and autumn than in males, suggesting preferential selection by females, possibly a result of differences in reproductive costs. Predation on earthworms was found to include deep-living species which only surface at night. This may have been a driving force in the evolution of nocturnal foraging behaviour in slow worms.

The prevalence of a parasitic nematode of slow worms, *Neoxysomatium brevicaudatum*, was also investigated using PCR primers. Prevalence was higher in males than females in April. While this might reflect different encounter rates between males and females, it may also be a function of testosterone-induced immunosuppression.

Analysis of smooth snake diet confirmed that predation on reptiles was high but revealed increasing consumption of small mammals with age. Predation on reptiles by grass snakes was higher than expected and suggests competition may exist between smooth snakes and grass snakes at a juvenile stage. Overall, these results provide a clearer picture of the trophic networks some of Britain's reptiles belong to, information pertinent to translocation and reintroduction programmes along with conservation management strategies.

*dedicated to my father*

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## **Chapter 1**

### **Introduction**

## 1.1 *Overview*

British reptiles are in rapid decline and surprisingly little is known about the reasons for this. Although habitat loss is clearly a factor, reptiles may also be indirectly affected by environmental changes that alter prey availability. As dietary preferences may change between juvenile and adult stages, availability of prey may be impacting upon reptiles at one or more developmental stages. The primary aim in this Thesis was therefore to develop new, non-invasive, molecular methods for dietary analysis of reptiles and apply it in the field. The first task was to establish whether molecular analysis of reptile faeces was even possible. Having established for the first time that it was, we went on to use prey-specific PCR primers, cloning and pyrosequencing to analyse predation by endangered smooth snakes (*Coronella austriaca*), grass snakes (*Natrix natrix*) and slow worms (*Anguis fragilis*). These techniques were used to investigate ontogenetic and seasonal changes in diet, and differences between males and females in prey selection. During this work it became apparent that nematode parasites were often present in slow worm faeces. As these may also have been affecting reptile fitness the project was expanded to include PCR-based analysis of nematode prevalence in the faeces of slow worms. Through large-scale ecological field studies this work aimed to develop a more detailed and comprehensive understanding of the trophic networks supporting reptiles and the potential importance of a range of prey species in their conservation.

## 1.2 *Background – the status of, and threats to, British reptiles*

Britain is home to just six species of reptiles. Its native reptile fauna consists of three snake species, the smooth snake (*Coronella austriaca*), the grass snake (*Natrix natrix*) and the

adder (*Vipera berus*), and three lizard species, the sand lizard (*Lacerta agilis*), common lizard (*Lacerta vivipara*) and slow worm (*Anguis fragilis*), all of which have declined in numbers over the past few decades as habitats continue to be lost, fragmented and unsympathetically managed. They are currently all protected under the Countryside and Wildlife Act 1981. It is illegal to kill, injure, harm or trade them, with additional legislation afforded to sand lizards and smooth snakes, which are particularly threatened.

Since the Industrial Revolution (1750-1850), the pressures of industrialization, mining, reptile collecting, urbanization and afforestation of land with conifers have had a massively damaging effect on native heaths, meadows and woodlands, preferred habitats for reptiles, yet the decline of these areas has been particularly apparent in the south of England. Between 1750 and 1960, Dorset lost 86% of its heathland (from 40,000ha to just 6,000ha) (Webb and Haskins 1980), the only habitat where all six species exist in high densities and the preferred habitat for all reptile species (Beebee and Griffith 2000). These heathlands now consist of just scattered and isolated pockets (Moore 1962; Spellerberg 1975).

Human activities have not only affected reptiles directly but have had adverse effects on the number, distribution and diversity of potential prey. Many vertebrate taxa found to be included in the diet of British snakes have experienced population declines.

Numbers of common shrews (*Sorex araneus*), pygmy shrews (*S. minutus*) and short-tailed voles (*Microtus agrestis*) have declined as grassland has become increasingly scarce (MacGillivray 1994), while the loss of ancient woodland has had an equivalent impact on yellow-necked mice (*Apodemus flavicollis*) and common dormice (*Muscardinus avellanarius*) (Harris and Woodland 1990). The water vole (*Arvicola amphibious*), abundant at the turn of the century in many lowland areas near water (Harris *et al.* 1995), has

subsequently endured a steady long-term decline (Strachan and Jefferies 1993) correlated with increased afforestation and resultant acidification of waterways (Harriman and Morrison 1982), and probably represents the most severe population decrease of any British mammal in the last century (White *et al.* 1997). This, combined with the effect of the introduced American mink (*Mustela vison*), is driving the water vole close to extinction (Woodroffe *et al.* 1990; Jefferies 2003). Other small mammal species which may have been components of snake diet in the past have been driven to extinction in Britain, such as the narrow-headed vole (*Microtus gregalis*) some 8,000-10,500 year B.P. and the root vole (*Microtus oeconomus*) just 1,500-3,500 year B.P. as a result of climate change and habitat loss (Clutton-Brock 1991). Amphibians, having aquatic and terrestrial stages in their development, are particularly sensitive to environmental changes in either, and are declining in numbers worldwide due to habitat loss (Brooks *et al.* 2002), introduced species (Adams 1999), pollution (Dunson *et al.* 1992) and pesticides (Relyea 2005), in addition to the effects of climate change (Pounds and Crump 1994). The common frog (*Rana temporaria*), common toad (*Bufo bufo*), natterjack toad (*B. calamita*) and great crested newt (*Triturus cristatus*) all suffered dramatic reductions of their UK populations throughout the 20<sup>th</sup> century ascribed to habitat loss (Cooke 1972; Beebee 1975; Beebee 1976), and in recent years, at least 50% of toad populations in the central and east/south eastern areas of England have experienced significant declines (Carrier and Beebee 2003). The pool frog (*Rana lessonae*), once considered a prey species of grass snakes (Blackwell Scientific UK Biodiversity Group Tranche 2. 1998) became extinct as recently as 1995 due largely to a reduction in the number and quality of suitable ponds in close proximity to one another (IUCN 2006). The sand lizard became extinct in Wales during the 1970s, and some 60% of

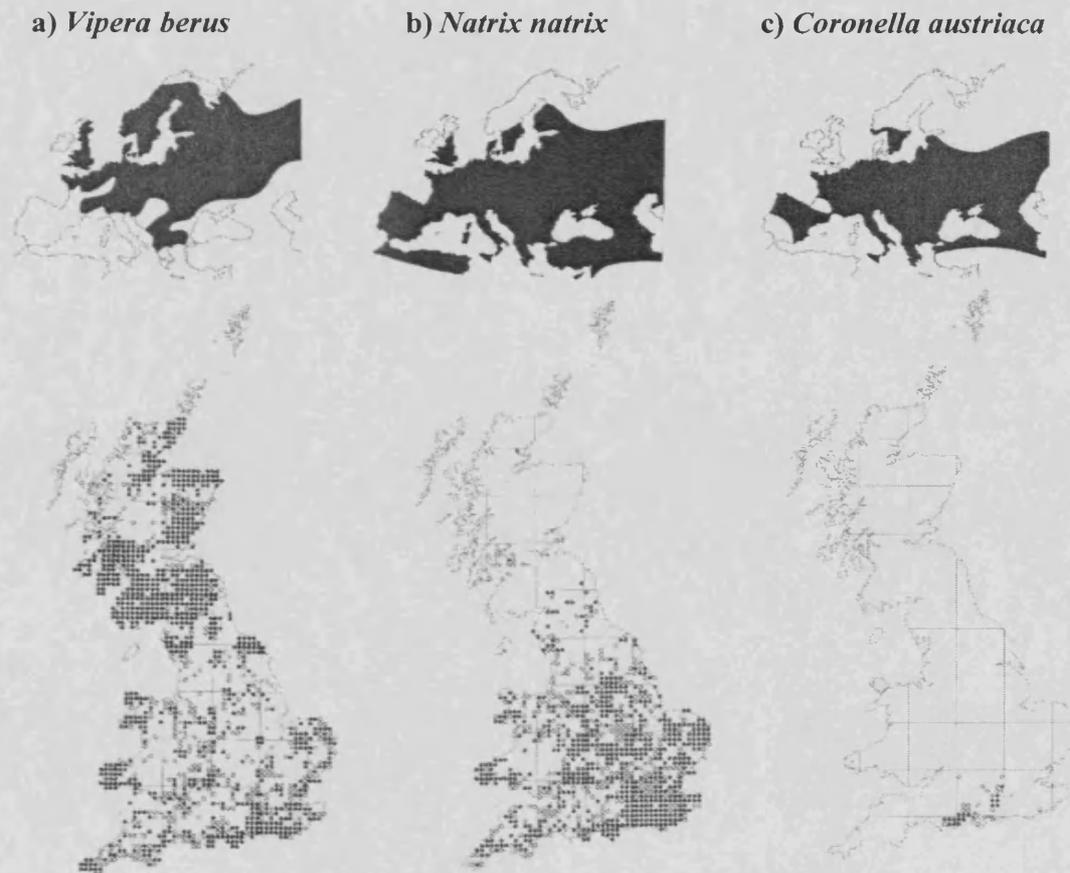
the once extensive Merseyside dune systems, which previously supported large sand lizard populations, disappeared to development during this time (Corbett and Moulton 1998). The devastation to the sand lizard population continued with the release of the myxomatosis virus in the 1950s which caused a collapse of the rabbit population and subsequently allowed trees and shrubs such as willow and sea buckthorn to colonise and replace large areas of dunes and heath, reducing optimal habitat for sand lizards (Corbett and Tamarind 1979). During the 1970s, reptiles had become very popular in the pet trade, and collecting of smooth snakes and sand lizards, both of which adapt readily to captivity, further decimated their populations and led to the extinction of sand lizards in the London heaths. Nesting birds have also been found in the diets of grass snakes (Brown 1991; Luiselli and Rugerio 1991), smooth snakes (Corbett in Nature Conservancy Council, 1983) and adders (Prestt 1971; Luiselli and Anibaldi 1990; Drobenkov 1995), and many bird populations have seen declines attributed largely to habitat loss, particularly of breeding grounds (Hilton-Brown and Oldham 1991; Donald *et al.* 2001; Vickery *et al.* 2001).

Snakes, particularly juveniles, may also include invertebrates in their diet (e.g. Spellerberg and Phelps 1977; Rugiero *et al.* 1995), while British lizards are all dependent on them (Avery 1962; Luiselli 1992; Beebee and Griffiths 2000; Pedersen *et al.* 2009). The population densities of a wide range of invertebrates continue to shrink, with many species already extinct (Dinnin and Sadler 1999) or on the brink of extinction (Thomas and Morris 1994). The effects on British reptile fauna of these reductions in prey are unknown, but are likely to be largely dependent upon the foraging strategies and degree of dietary specialization.

### *Distribution of British Reptiles*

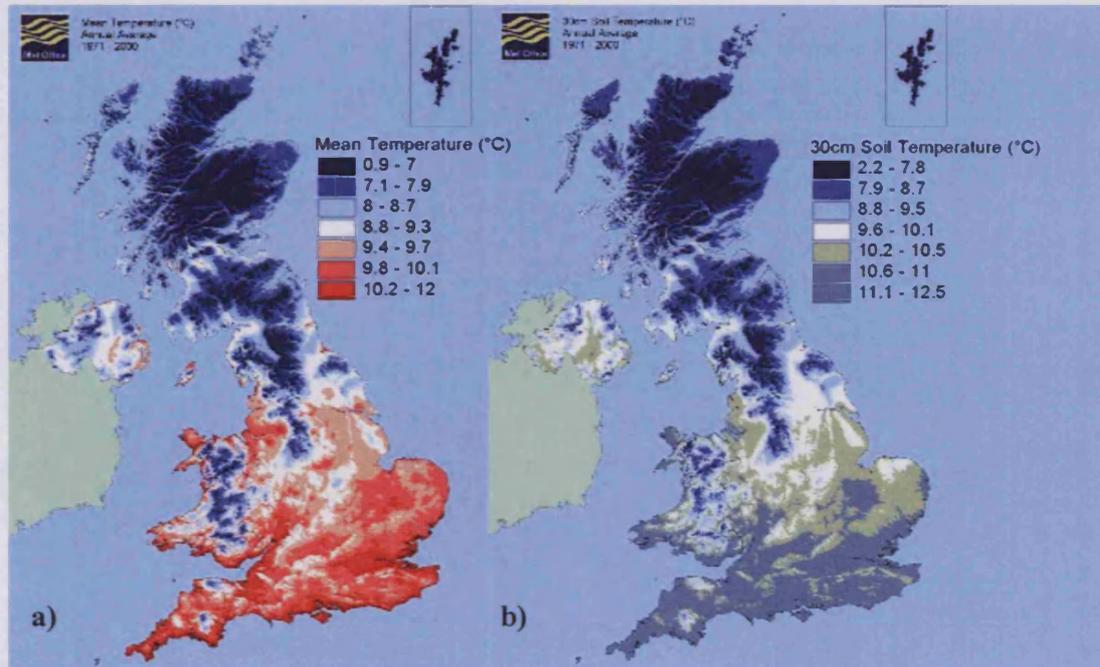
Britain experienced its warmest climate since the last Ice Age between 7,000 – 5,000 years B.P., during which time it became isolated from mainland Europe (Barnekow 2000; Korhola *et al.* 2000; Seppa and Birks 2002). Sometime shortly before then a north-westerly invasion of reptiles must have occurred with species traveling across the land bridge into East Sussex and Kent before migrating northwards. The temperature in Britain dropped by 3-4°C between 3,000 and 2,500 years B.P. from which we have yet to fully recover (Johnson and Smith, 1965; Rousseau, Preece and Limondin-Lozouet, 2010). Forest communities became replaced by heath communities (Conway 1947) and reptiles are likely to have spread into the northwest as habitats became favourable, receding southwards again when temperatures dropped.

While all of Britain's reptiles are in decline and exist in fragmented populations, their distributions differ greatly. Amongst the lizards, the common lizard and slow worm are widespread and locally abundant across the United Kingdom, whereas the sand lizard is more specialised in its habitat requirements, requiring areas with at least 1-5% of open exposed sand for egg laying (Corbett and Tamarind, 1979), and persists now in just two areas of coastal dunes or lowland heath, in southern England and in Merseyside (Corbett 1988). The distributions of Britain's snakes are also markedly different to one another. The adder is found in a wide range of habitats throughout Britain and extends from the southern coast of England to the northern coast of mainland Scotland (Reading *et al.* 1996) (Fig. 1.1). whilst the grass snake is restricted to England and the smooth snake has a restricted distribution in the south (Fig. 1.1).



**Figure 1.1** Distribution of British snakes in the UK and throughout Europe (taken from Beebee and Griffiths 2000).

The grass snake, a colubrid and Britain's only oviparous snake, is locally abundant and widespread across England and Wales but rare above 56°N (Fig. 1.1b), equating approximately to the English-Scottish border and implying that their distribution is largely governed by temperature. They are mostly absent in areas where the average annual temperature is below 9.4°C (Fig. 1.2a) and, possibly more relevant, where the minimum grass temperature is below 10.2-10.5 °C (Fig. 1.2b).



**Figure 1.2** a) Annual mean temperature (°C) between 1971-2000, and b) Annual mean soil temperature (°C) 1971-2000 (Met Office).

The adder's widespread occurrence across Britain, particularly its presence in the most northern latitudes (Fig. 1a), may partially be explained by its efficient thermoregulatory behaviour (Spellerberg 1975), but it may also be a result of its diverse feeding ecology, with adders able to survive on a more diverse range of prey than the other British snakes (Bell 1869; Prestt 1971; Andren and Nilson 1983; Drobenkov 1995) and therefore diet is less likely to be restrictive on their distribution. The smooth snake, while reproductively similar to the adder (they are both viviparous, breed every 2-3 years and produce a similar number of young), and of equivalent size and with overlapping diets (Prestt 1971; Goddard 1984), has a considerably different distribution, with populations restricted to a small number of hotspots in the south of England (Fig. 1.1c). It has been suggested that their distribution, like

grass snakes, is constrained by temperature (Spellerberg and Phelps 1977); however, this does not fully explain why smooth snakes have a restricted distribution in certain regions of Hampshire and Dorset only, when they are found to range much farther north in Europe (Gasc 1997). As they are found only on sandy lowland heaths in the UK it is commonly believed that this habitat is structurally or ecologically important and represents a limiting resource for smooth snakes (Braithwaite *et al.* 1989). Why this should be the case in Britain though, when they are found in a variety of habitats throughout continental Europe (pine forests, mixed riverside forests and vegetation bordering fields, shrubs, orchards (e.g. Luiselli and Capizzi 1997)) is not clear. An alternative is that smooth snake distribution is a function of diet, prey availability, prey diversity and competition with other predator's including adders and grass snakes for food (Drobenkov, 1995; Goddard, 1984).

Slow worms have a widespread, locally abundant distribution (Fig. 1.3) in Britain and are found in an array of different habitats including rough grasslands, hedgerows, heathland, woodland edges, downs and moorland, gardens, churchyards, parks, allotments, motorway embankments (Griffith and Beebee 2000). While their diet has been relatively unstudied, a couple of studies have revealed they consume predominantly earthworms and molluscs (Luiselli 1992; Pedersen *et al.* 2009), a ubiquitous prey in the UK. It is unclear, therefore, why some apparently suitable areas are devoid of slow worm populations and apparently unable to support them.



**Figure 1.3.** Distribution of the slow worm, *Anguis fragilis*, in the UK and throughout Europe (from Beebee and Griffiths 2000). Solid and open circles are post- and pre-1970 records respectively.

#### 1.4 *Diet of British reptiles*

All three species of snake have similar periods of annual activity, emerging from their hibernacula after five months between October and March, spending a few weeks basking before mating, and only then heading to the summer feeding sites in search of prey (Beebee and Griffiths 2000.).

Our present understanding of snake diet originates from a number of different methodologies: direct observations (Spellerberg 1977), correlations with distributions of potential prey (Andren and Nilson 1976, 1979; Spellerberg and Phelps 1977), post-mortems of the gut (Prestit 1971; Drobenkov 1995) and more commonly analysis of regurgitates (Goddard 1984; Spellerberg and Phelps 1977; Drobenkov 1995; Reading and Davies 1996; Luiselli and Anibaldi 1990; Gregory and Isaac 2004) or faeces (Spellerberg and Phelps 1977; Rugiero *et al.* 1995), each of which are prone to biases towards species that are larger, more visual or which have hard body parts.

#### 1.4.1 *Grass snake (Natrix natrix) diet*

The grass snake has no special adaptations for subduing their prey, instead simply darting out and grabbing their prey with their recurved teeth to hold it firmly while they swallow it alive. This limits their ability to handle larger prey that adders and smooth snakes are capable of. They are highly aquatic and generally accepted to be specialist predators of amphibians (particularly anurans) (Table 1.1) with their preferred habitats of ponds, lakes, marshes and streams reflecting this. In one study, 62.9% of the amphibians contributing to their diet were common frogs (Drobenkov 1995) despite eight other amphibian species being regularly eaten, while in another study 97.6% of the diet comprised of common toads (Reading and Davies 1996). It is likely that differences in prey availability between the sites in these studies are responsible for these findings. Reading and Davies (1996) calculated that grass snakes consume a toad on average once every 20 days, with males having an average consumption of 8.1 toads per year and females 5.5 toads per year. Other prey items found to

	AMPHIBIANS			SMALL MAMMALS						REPTILES					FISH	BIRDS		
	Anurans	Urodeles	Total	Shrews	Voles	Rodents	Rodents (nestling)	Nestling (sp. unident.)	Total	Snakes	Sand Lizards	Common Lizards	Slow Worms	Lizards (any)				Total
A	147	6	153	-	-	-	-	-	5	-	-	-	-	-	-	3	2	Grass snake ( <i>Natrix natrix</i> )
	<b>90.2</b>	<b>3.7</b>	<b>93.9</b>						<b>3.1</b>							<b>1.8</b>	<b>1.2</b>	Stebbing unpubl. (U.K.) in PTG
A	40	-	40	-	-	-	-	-	-	-	-	-	-	-	-	1	-	Reading and Davies, 1996 (U.K.)
	<b>97.6</b>		<b>97.6</b>													<b>2.4</b>		
Z	24	2	26	-	-	-	-	-	-	-	-	-	-	-	-	-	1	Brown 1991 (U.K.)
	<b>88.9</b>	<b>7.4</b>	<b>96.3</b>														<b>3.7</b>	
B	68	-	68	-	-	-	-	-	-	-	-	-	-	-	-	6	-	Madsen 1983 (Sweden)
	<b>91.9</b>		<b>91.9</b>													<b>8.1</b>		
B	191	-	191	-	-	-	-	-	1	-	-	-	-	-	1	10	2	Luiselli and Rugiero, 1991 (Italy)
	<b>93.2</b>		<b>93.2</b>						<b>0.5</b>						<b>0.5</b>	<b>4.9</b>	<b>1.0</b>	
B	11	-	11	-	-	-	-	-	-	-	-	-	-	-	1	-	-	Capula <i>et al.</i> 1994 (Italy)
	<b>91.7</b>		<b>91.7</b>												<b>8.3</b>			
B	52	-	52	-	-	-	-	-	1	-	-	-	-	-	2	8	-	Filippi <i>et al.</i> 1996 (Italy)
	<b>82.5</b>		<b>82.5</b>						<b>1.6</b>						<b>3.2</b>	<b>12.7</b>		
B	162	21	183	-	-	-	-	-	6	-	-	-	-	-	1	-	-	Luiselli and Capula, 1997 (Italy)
	<b>85.3</b>	<b>11.1</b>	<b>96.3</b>						<b>3.2</b>						<b>0.5</b>			
A	129	3	132	-	-	-	-	-	-	-	-	3	-	-	3	6	-	Drobenkov 1995 (Lithuania & Ukraine)
	<b>91.5</b>	<b>2.1</b>	<b>93.6</b>									<b>2.1</b>			<b>2.1</b>	<b>4.3</b>		

**Table 1.1.** Dietary composition of grass snakes (*Natrix natrix*). Shown are numbers of snakes containing prey (A), total number of prey items (B), and uncertain or mixed data (Z), with percentages represented in bold. Country of study is shown in parentheses.

be occasionally taken include small mammals, common lizards and, while less common, birds, large slugs (Jonty Denton unpubl. data, reported in Beebee), and even bees (Smith 1951), although these supplementary prey are generally not considered as important features of their diet. Where small mammals are taken, they are largely nestling rodents, although shrews have been found on occasion. With females growing to be around 80cm compared to 65cm for males, diet may potentially differ between sexes as a function of size (Luiselli and Capula 1997), a suggestion supported by some (Luiselli *et al.* 2005) but rejected by others (Reading and Davies 1996). There is an ontogenetic change in their diet, with that of juveniles consisting of small amphibians and fish broadening as snakes grow bigger and become capable of taking larger prey (Luiselli and Rugiero 1991; Madsen 1983; Luiselli *et al.* 1997; Reading and Davies 1996, Gregory and Isaac 2004). It has been suggested that the young may also feed on worms and slugs (Reading and Davies 1996) although there is no direct evidence of this due to difficulties of detecting consumption of soft-bodied prey.

#### 1.4.2 *Smooth snake (Coronella austriaca) diet*

Britain's other colubrid, the smooth snake, is a partial constrictor which coils around its prey to subdue it. Its diet is less certain than that of grass snakes and adders (Table 1.2). Considered to be a dietary specialist feeding almost exclusively on reptiles throughout its continental European range (Andren and Nilson 1976, 1979; Duguy 1961; Bruno 1966; Drobenkov 1995), reports on UK populations is more uncertain, with general agreement over the main range of prey but disagreement over the importance attributed to each. From collating various reports of smooth snake diet collected in England, Corbett (in Nature

	AMPHIBIANS			SMALL MAMMALS					REPTILES					FISH	BIRDS			
	Anurans	Urodeles	Total	Shrews	Voles	Rodents	Rodents (nestling)	Nestling (sp. unident.)	Total	Snakes	Sand Lizards	Common Lizards	Slow Worms				Lizards (any)	Total
A	-	-	-	13	-	-	2	12	27	-	-	14	-	-	14	-	-	Smooth snake ( <i>Coronella austriaca</i> )
				<b>31.7</b>			<b>4.9</b>	<b>29.3</b>	<b>65.9</b>			<b>34.1</b>			<b>34.1</b>			Goddard 1984 (U.K.)
B	-	-	-	23	-	-	2	23	48	-	-	15	-	-	15	-	-	Goddard 1984 (U.K.)
				<b>36.5</b>			<b>3.2</b>	<b>36.5</b>	<b>76.2</b>			<b>23.8</b>			<b>23.8</b>			
A	-	-	-	-	2	-	-	-	2	3	4	18	2	-	27	-	-	Drobenkov 1995 (Lithuania & Ukraine)
					<b>6.9</b>				<b>6.9</b>	<b>10.3</b>	<b>13.8</b>	<b>62.1</b>	<b>6.9</b>		<b>93.1</b>			
B	-	-	-	-	-	4	-	-	4	1	-	-	1	37	39	-	-	Rugiero <i>et al.</i> 1995 (Italy)
						<b>9.3</b>			<b>9.3</b>	<b>2.3</b>			<b>2.3</b>	<b>86.0</b>	<b>90.7</b>			
Z	1	-	1	1	1	-	10	-	14	5	13	7	1	2	28	-	5	Corbett (in Nature Conservancy Council 1983) (U.K.)
	<b>2.1</b>		<b>2.1</b>	<b>2.1</b>	<b>2.1</b>		<b>20.8</b>		<b>29.2</b>	<b>10.4</b>	<b>27.1</b>	<b>14.6</b>	<b>2.1</b>	<b>4.2</b>	<b>58.3</b>		<b>10.4</b>	

**Table 1.2.** Dietary composition of smooth snakes (*Coronella austriaca*). Shown are numbers of snakes containing prey (A), total number of prey items (B), and uncertain or mixed data (Z), with percentages represented in bold. Country of study is shown in parentheses.

Conservancy Council, 1983) revealed far greater diversity of diet than previously thought, identifying at least nine different prey species. He calculated that 58% of their diet consisted of reptiles (sand lizards, common lizards, slow worms, and juvenile and immature adders, grass snakes and even other smooth snakes) and 29% consisted of mammals. Birds and anurans made up 10.4% and 2.1% of the diet respectively. The data from these studies originated from a variety of different methods: faecal analyses, gut analyses, post-mortems and direct observations. Presumably data based on direct observations will be biased towards reptiles while underestimating predation on nestling mammals (as nestling mammals are often in underground burrows), and it is interesting to note that if all observed data is excluded from Corbett's report then the results suggest that mammals and reptiles are equally important, with each making up 46.4% of the total diet. Goddard (1981, 1984) also found a high proportion of mammals in UK populations. His analysis of prey remains in faecal samples revealed 53% had eaten small mammals and 48% had eaten reptile. From his analysis of regurgitates he found that the proportion of smooth snakes which had consumed mammals was twice that of those that had consumed reptiles. Goddard (1984) suggested there was no evidence for specialism on either lizards or small mammals and instead speculated they consumed prey in accordance with their encounter rate with different prey. This is supported by Rugeiro *et al.* (1995) who found, using faecal and regurgitate analyses of smooth snakes in Italy, that they were eating prey in accordance with the ratio of prey availability. However, an innate feeding preference for lacertid lizards was found in juveniles during captive studies (Goddard 1984), indicating smooth snakes may initially be restricted to lizards, broadening their diet with increasing age and size. At an even younger age they may be restricted to an invertebrate diet, with a number of researchers reporting an

insect component to the diet (Corbett in Nature Conservancy Council 1983; Spellerberg and Phelps 1977; Rugiero *et al.* 1995), although such reports are largely anecdotal and do not consider that any invertebrates found may be the result of secondary predation by another prey species rather than direct predation.

### 1.4.3 *Adder (Vipera berus) diet*

Of the terrestrial snakes, the adder is one of the most geographically widespread worldwide (Steward, 1971). It is a viper, using venom to pacify or kill their prey. It has the maximal food diversity index of any snake worldwide (Drobenskov 1995), although principally they prey upon small mammals (Table 1.3), with an estimated consumption of the equivalent of nine field voles per year (Andren and Nilson 1983). Smith (1951) first suggested common lizards as the chief component of their diet, following captive studies where he found a preference for common lizards and slow worms over anurans and baby mice. Most studies, however, have found small mammals to be their favoured prey in the wild (Bell, 1869; Prestt 1971; Andren and Nilson 1983; Drobenskov 1995). This discrepancy in findings may arise from the age classes of individuals being investigated. Prestt (1971) claimed there were ontogenetic changes in diet, finding that the prey of young adders comprised lizards and small mammals but that once snakes were above 30cm in length there was a marked switch to mostly mammalian prey. By determining the gut contents of adders (adults and juveniles) through post-mortem analysis, Prestt (1971) found that 91% of the prey of adults were mammals (9% nestling mammals, 82% adult mammals) compared with 69% of juveniles (31% nestling mammals, 38% adult mammals). Only 5% of the adults included lizards in their diet, compared with 31% in juvenile diet. Prestt's (1971) study also demonstrated just

	AMPHIBIANS			SMALL MAMMALS					REPTILES					FISH	BIRDS			
	Anurans	Urodeles	Total	Shrews	Voles	Rodents	Rodents (nestling)	Nestling (sp. unident.)	Total	Snakes	Sand Lizards	Common Lizards	Slow Worms				Lizards (any)	Total
B	1	-	1	10	24	15	-	-	49	-	-	-	-	-	-	-	5	<b>Adder (<i>Vipera berus</i>)</b>
	<b>1.8</b>		<b>1.8</b>	<b>18.2</b>	<b>43.6</b>	<b>27.3</b>			<b>89.1</b>								<b>9.1</b>	<b>Luiselli and Anibaldi 1990 (U.K.)</b>
A	8	-	8	11	32	5	-	-	48	-	-	10	1	-	11	-	3	<b>Drobenkov 1995 (Lithuania &amp; Ukraine)</b>
	<b>11.4</b>		<b>11.4</b>	<b>15.7</b>	<b>45.7</b>	<b>7.1</b>			<b>68.6</b>			<b>14.3</b>	<b>1.4</b>		<b>15.7</b>		<b>4.3</b>	
B	-	-	-	20	35	8	-	16	63	-	-	11	2	-	13	-	5	<b>Prestt 1971 (U.K.)</b>
				<b>24.7</b>	<b>43.2</b>	<b>9.9</b>		<b>19.8</b>	<b>77.8</b>			<b>13.6</b>	<b>2.5</b>		<b>16.0</b>		<b>6.2</b>	

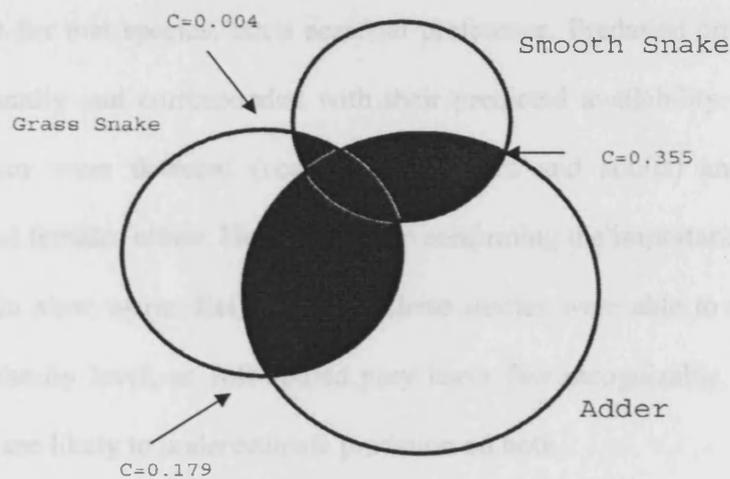
**Table 1.3.** Dietary composition of adder (*Vipera berus*). Shown are numbers of snakes containing prey (A) and total number of prey items (B), with percentages represented in bold. Country of study is shown in parentheses.

how diverse the diet of adult adders was, finding that it included birds, common lizards, slow worms, long-tailed field mice (*Apodemus sylvaticus*), harvest mice (*Micromys minutus* Pallas), common shrews, pygmy shrews, water shrews, and short-tailed voles. Prestt (1971) also noted that some vertebrate species present in the area were not found to be taken by adder, these included the common toad, sand lizard, water vole and mole (*Talpa europaea*), although relative abundance of the different species present was not considered. A comprehensive study in Lithuania and Ukraine (Drobenkov 1995) reported the same diverse diet for the adder but with 80% of their diet consisting of small mammals (from at least six species).

#### 1.4.4 *Diet overlap amongst snakes*

In Drobenkov's (1995) study of snake diet in Lithuania, where all three snake species live in sympatry, the same trends in diet were found as suggested for Britain, with grass snakes preying mostly upon amphibians, smooth snakes upon reptiles and adders predominantly favouring small mammals. There was little overlap in the diet of grass snakes and smooth snakes, with the only shared prey being slow worms, which was not a predominant component of either's diet (2.1% and 6.9% respectively). Using the Morisita index (Morisita 1959), which varies from zero (when species have no overlap) to one (when diets are identical) the result of  $C=0.004$  testified to their separate dietary niches. However, there was considerably greater overlap between the diet of the adder with grass snakes ( $C=0.179$ ) and smooth snakes ( $C=0.355$ ). Adders and grass snakes shared four prey species, equating to 84% of the grass snakes' diet and 13% of the adders'. Adders and smooth snakes only overlapped in their consumption of slow worms, common lizards and voles, which

comprised 76% of the smooth snakes' diet and 36% of the adders' (Drobenkov 1995). If this overlap of resources (Fig. 1.4) is similar in Britain, where there are fewer prey species and lower densities of them, it could represent considerable competition between adders and the other snake species, in particular smooth snakes.



**Figure 1.4.** Overlap of the diet of smooth snakes, adders and grass snakes using the Morisita Index (Morisita 1959) from Drobenkov (1995). Size of circles represents diet breadth.

#### 1.4.5 *Slow worm (Anguis fragilis) diet*

Establishing the diet of slow worms by direct observation is impossible, as they are an elusive and semi-fossorial reptile. Of 24 slow worms examined by gut dissection in Italy,

35% were found to contain pulmonates and 33% earthworms, with other prey, diptera, lepidoptera, coleopteran, homoptera, and araneidae, all present in less than 10% of each animal. Through analysis of the faeces and regurgitates of 84 slow worms in Denmark, 44% were found to contain pulmonates, 22% millipedes (belonging to one species, *Glomeris marginata*), and 21% earthworms. Other millipede species were present, but were not consumed, and predation on *G. marginata* was significantly higher in the spring than in the rest of the year despite the abundance of them increasing throughout the year, suggesting not only a preference for that species, but a seasonal preference. Predation on earthworms did not change seasonally and corresponded with their predicted availability. No ontogenetic differences in prey were detected (comparing juveniles and adults) and no difference between males and females either. However, while confirming the importance of pulmonates and earthworms in slow worm diet, neither of these studies were able to identify them to species or even family level, as soft-bodied prey leave few recognizable remains, and as such the findings are likely to underestimate predation on both.

### 1.5 *Understanding and measuring trophic interactions*

Trophic interactions are one of the key influences on populations dynamics and are therefore a fundamental ecological consideration. Looking at single predator-prey interactions in isolation can be misleading when often species are involved in a web of direct and indirect interactions (Pianka 1987). To fully understand the diversity, complexity and stability of ecosystems, piecing together food-webs is crucial. A better knowledge of these complex networks can be useful in addressing practical environmental issues, such as developing strategies for integrated pest management or for wildlife conservation, where it is important

to be able to make realistic predictions of the potential consequences of modifying a food-web. They can be used, for instance, to predict the community level impacts of biological controls, habitat management, climate change, pollution, introduced alien species or reintroduction of species to native habitats (Forup *et al.* 2008). In such cases, it is important to consider the interactions between species, and not just whether they are present / absent (Ehrenseld and Toft 1997). The availability of prey is often the most important mechanism affecting predator distribution, with the ideal free distribution theory stating that a predator's distribution will reflect that of its prey (Fretwell and Lucas 1970; Fretwell 1972). Therefore, understanding a predator's distribution can be dependent more upon a sound knowledge of its prey choices than on its habitat preferences.

British snakes and slow worms appear to be generalist predators, all having broad diets but showing some degree of specialism / preference. This is particularly evident in the different prey and ratios of prey found in their diets by different studies, presumably the result of a disparity in prey availability of the different study sites. Generalists often only feed opportunistically when prey availability is suboptimal, usually exhibiting preferences in prey choice affected by: nutritional value, prey defenses (mechanisms, toxins and escape proficiency), ratio of available prey, time since feeding, intra-, inter-specific and apparent competition, and the population densities and spatial and temporal distributions of predators and prey (Symondson 2002). When densities of preferred prey are low, predators may be forced to change their foraging mode or switch prey, in order to maximize their overall rate of energy intake, depending on the availability of alternative prey and the time spent foraging for and handling it (MacArthur and Pianka 1966). The Alternative Prey Hypothesis predicts that a generalist predator with a strong preference for a main prey will shift its diet

to alternative prey when the main prey is scarce and as such the mortality rate of alternative prey should be inversely correlated to the abundance of main prey (Hagan 1952; Lack 1954; Hornfieldt 1978; Angelstam *et al.* 1985; Small *et al.* 1993). Not only may alternative prey be essential to the persistence of a generalist predator, whose main prey goes through population cycles, but a high diversity of prey can increase a generalist predator's fitness, by satisfying nutritional requirements (Greenstone 1979; Mayntz *et al.* 2005) and enhancing fecundity and growth rate (e.g. Toft and Wise 1999; Oelbermann and Scheu 2002; Symondson *et al.* 2006; Harwood *et al.* 2009), and therefore prey diversity may actually be more important to a generalist than the abundance of any individual prey species.

For many predators, diet changes seasonally, due to fluctuations in prey availability (e.g. Slip and Shine 1988; Houston and Shine 1993; Santos *et al.* 2000), and ontogenetically, commonly as a result of improved foraging (Rutz *et al.* 2006) or the range of prey available increasing with predator size (Dickman 1988). Additionally, diet may also differ between males and females due to sexual dimorphism (reviewed in Shine 1989) or behaviour driven by differential nutritional needs or differences in habitat use affecting encounter rates with prey (e.g. Pyke, Pulliam and Charnov 1977; Ryan, Bartholomew and Rand 1983; Savitsky 1983). Therefore it is necessary to consider the entire life history of a species to gain a comprehensive view of a predator's place within a network.

Despite their influence in structuring and stabilizing networks, parasites and parasitoids are frequently ignored or considered in isolation, with ecologists often restricting their studies to predator-prey interactions only (Lawton 1989). Yet almost all species are prone to parasitism (e.g. Esch and Fernandez 1993; Dobson *et al.* 2006). While the effects of parasites are usually intensity-dependent and do not always involve host death, in parasitoid-

host interactions one host is killed by one prey and therefore their impact can be comparable to that of a predator's. As such they can have a major influence on population dynamics (e.g. Synder and Ives 2003) and have been used as biological controls in manipulating food-webs and controlling pest species (e.g. Bustillo and Drooz 1977; Foster and Luck 1996). While there is occasionally inclusion of parasites in food-webs (e.g. Husham *et al.* 1996; Thompson *et al.* 2005; Lafferty *et al.* 2006a), their role is seldom addressed, yet parasites may have a large influence on a host's trophic interactions through effects on energetic demands, nutritional status or growth rates (reviewed in Minchella and Scott 1991). Parasites may also be transmitted through food-webs, sometimes via trophic interactions themselves (Minchella and Scott 1991). Some of those that are transmitted trophically have an affect upon the behaviour of the host (Moore 2002) and can dramatically increase the strength of trophic interactions as well as creating new ones. For example, a trematode which infects killifish in California modifies behaviour of the intermediate host such that they are 10-30 times more likely to be eaten by birds, which serve as final hosts to the parasite (Lafferty and Morris 1996).

To construct an accurate picture of these potentially complex ecological networks, appropriate methods for measuring trophic interactions are necessary. Direct observation can reveal foraging behaviour and important aspects of diet, but often it can be difficult, time-consuming and unfeasible to corroborate trophic interactions by these means, particularly with respect to highly mobile, small, elusive, cryptic or rare species (Sheppard and Harwood 2005). Alternatively, predation can be inferred by analysis of gut dissections, faeces, or regurgitates using morphologically recognizable features of prey remains to allow identification of specific prey taxa. The attraction of such approaches is that an animal's

feeding behaviour is not disturbed and information gained represents a snapshot of its diet without the biases toward certain prey that direct observations are likely to have (Sunderland 1988; Harwood and Obrychi 2005). Whereas invertebrate studies sometimes rely on visual examinations following gut dissections (reviewed in Ingerson-Mahar, 2002), with vertebrates noninvasive techniques dominate, such as the analysis of regurgitated bird pellets (eg., Pavey and Burwell, 1997; Elmhagen *et al.* 2000) although more invasive approaches are occasionally utilized, such as gut dissections (reviewed in Pierce and Boyle, 1991) or stomach pumping (Waltr and O'neill, 1986). However, while some indigestible remains may contain distinguishable features allowing visual identification in the guts, faeces or regurgitates of predators (such as cuticles, bones, scales, fur or features) which may provide useful information as to the prey eaten, digestive processes damage many diagnostic features, and soft-bodied prey leave no recognizable remnants at all. Hence these approaches are prone to biases and may miss trophic links altogether (Dennison and Hodkinson 1983) leading to overestimation of predation on large species or those with robust hard parts (Harvey 1989; Tollit *et al.* 1997) and underestimation of predation on small and soft-bodied prey. The accuracy of this approach also depends on the investigator's ability to distinguish prey from their remains, and differences in these abilities between investigators will influence comparative studies (Admassu *et al.* 2006). As a result of these discrepancies and limitations a number of techniques have been developed aimed at identifying prey from molecular signatures such as species-specific protein sites or DNA sequences (reviewed in Symondson 2002, King *et al.* 2008). In addition to potentially eliminating the inherent biases and constraints present in visual observation these techniques are less disruptive to the study organisms. Furthermore, they can be applied to investigating trophic links of parasites

(Small *et al.* 2007), parasitoids (Traugott *et al.* 2008) and micropredators (Lueders *et al.* 2006) which are otherwise difficult to measure due to their size (often microscopic) and elusive nature.

### 1.6 *Molecular techniques for the detection of prey*

There are a number of molecular techniques currently available (e.g., radioisotope labeling, electrophoretic detection of prey isozymes, reviewed by Sunderland 1988, 1996; Pierce and Boyle 1991; Greenstone 1996; Symondson 2002) but current studies have focused on the use of antibody and / or DNA-based techniques which allow rapid screening of predator guts / faecal samples for prey remains.

The use of polyclonal antibodies was first described by Brooke and Proske (1946) in studying predation on mosquitos, although their lack of specificity (Miller 1979) led to them being superseded by monoclonal antibodies (reviewed in Symondson 2002). This can be used in detecting predation on groups (e.g. Symondson *et al.* 1999b), individual species (e.g. Hagler *et al.* 1993; Symondson and Liddell 1996; Symondson *et al.* 1997), stages (e.g. Symondson *et al.* 1999; Hagler *et al.* 1992; Crook and Solomon 1996; Symondson and Liddell 1993), or even specific instars (Greenstone 1996; Symondson 2002). In addition to their high specificity, once created, monoclonal antibodies are inexpensive to propagate and samples can be assayed cheaply, easily and very rapidly, with prey antigens sometimes having long detection periods following their consumption (eg. Harwood *et al.* 2001; Schenk and Bauer, 2004). Where large numbers of predators are to be analysed through mass-screening for specific prey the simplicity of the screening protocols has made monoclonal antibody-based assays the most viable approach (e.g. Harwood, Sunderland and Symondson,

2004; Hagler and Naranjo, 2005). However, it can take up to a year to develop antibodies that can detect epitopes which resist digestion for any practical length of time while maintaining specificity (Symondson 1999), and investigating the dietary breadth of a generalist, which would require numerous monoclonal antibodies to target different prey, would therefore be unrealistic.

DNA-based techniques are now favoured because of the considerable cost of antibody production along with the time-consuming and technically challenging development of them. The use of PCR in studying diet was first demonstrated by the identification of bloodmeals consumed by haematophagous insects (Couldson *et al.* 1990; Tobolewski *et al.* 1992; Gokool *et al.* 1993) and first applied to faecal analysis in the detection of plant DNA in the diet of bears (Hoss *et al.* 1992). Zaidi *et al.* (1999) suggested that targeting multicopy DNA would improve detectability, which was corroborated in subsequent experiments (Chen *et al.* 2000; Agusti and Symondson 2001). Although the use of multiple copy nuclear DNA has proved successful, such as nuclear ribosomal RNA genes (e.g. Hoogendoorn and Heimpel 2001) which contain hundreds or thousands of repeats in the eukaryote genome, the use of mitochondrial DNA has dominated. Mitochondrial DNA is present in diploid cells in thousands of copies compared to the two copies of nuclear DNA, the systematic relationships are well understood (Simon *et al.* 1994; Caterino *et al.* 2000) and there is an abundance of sequence data for an enormous number of taxa available on public databases such as Genbank and BOLD. The size of the target DNA amplicon also determines its detectability, with longer fragments degrading rapidly (Zaidi *et al.* 1999; Chen *et al.* 2000; Hoogendoorn and Heimpel 2001; Agusti *et al.* 2003) and amplicons below 300 base pairs have generally been favoured. These PCR techniques have been successfully

applied to studying the diet of a wide number of invertebrates including beetles (e.g. King *et al.* 2010), spiders (e.g. Agusti 2003b) and vertebrates (such as fish (e.g. Sailoh *et al.* 2003) and mammals (e.g. Deagle *et al.* 2005)). The use of group-specific or universal primers, first used by Jarman *et al.* (2002) on exploring the diet of pygmy blue whales and adèle penguins, allow the amplification and sequencing of DNA for prey species for which there is no DNA sequence information available for primer-design (Simon *et al.* 1994) or no prior knowledge of a predator's/herbivore's diet. Once prey DNA has been amplified in PCR, there have been a range of approaches used to identify the amplicons based on species-specific fragment size (for example by fragment analysis (e.g. Harper *et al.* 2005) or PCR-RFLP (e.g. Parsons *et al.* 2005)) or by species-specific chemical properties (i.e. DGGE (e.g. Deagle *et al.* 2005)), or melting temperature (i.e. TGGE (Harper *et al.* 2006)) of the amplicon. More commonly, amplicon identity is established by sequencing after cloning (e.g. Deagle *et al.* 2007). The latter technique is being superseded by the use of new generation high-throughput sequencing (e.g. Deagle, Kirkwood and Jarman 2009), currently capable of producing up to 600 million base pairs in a single run with 400-500 base pair reads. Overall, where the aim is to identify a range of species, DNA-based approaches have numerous advantages: The techniques to develop primers are widely known and facilities widespread; commercial kits are available making the techniques simple even for non-molecular scientists; there are banks of sequence data available publicly for thousands of species; and, once species-specific primers are developed they can be cheaply manufactured for use in reproducible protocols.

**1.7 Aims**

The aim of this study was to develop and apply DNA-based approaches to analysis of the diet, parasites and trophic interactions of British reptiles, with particular focus on the smooth snakes, Britain's most endangered reptile, and the slow worm, Britain's least understood reptile. Specifically, the aims were:

1. To determine whether PCR is a practical tool for the analysis of reptile diets from faecal samples.
2. To examine the individual species of pulmonates and earthworms being consumed by slow worms in order to investigate: ontogenetic, seasonal and sex-based differences in prey selection, look at abiotic (e.g. weather, location) and biotic (e.g. body size) influences upon diet, and to determine the diversity of prey consumed.
3. To determine the prey of smooth snakes and grass snakes using specific primers for a suite of prey in order to investigate ontogenetic, seasonal and sex-based differences in prey selection, differences in prey taken by grass snakes and smooth snakes and the potential for competition between them.
4. To establish whether pyrosequencing is more rapid and practical means of determining reptile diets.
5. To investigate parasite prevalence in slow worms, whether it is sex-biased, and what factors appear to influence it.

A more comprehensive picture of trophic interactions between these predators and their prey may offer a valuable insight into their present distributions and possible constraints upon

them, useful in conservation management, reintroduction programmes and appropriate translocations of populations prior to land development.

## 1.8 *Thesis layout*

In Chapter 2 various method development and preliminary analyses are described. Details of species-specific PCR primers designed to target certain prey are given; and of universal primers tested against a wide range of taxa to find primers suitable for a cloning/pyrosequencing approach. Experiments on captive garter snakes and slow worms demonstrate that prey DNA is detectable in reptile faeces, and suggest prey may remain detectable for longer in snakes than in slow worms. Faeces from wild slow worms are amplified with general earthworm primers and cloned and sequenced to identify prey species. This establishes the practicality of this approach before a more thorough analysis of slow worm diet using general earthworm primers and pyrosequencing in Chapter 4.

Presented in Chapter 3 are the attempts to develop a method for preventing predator DNA from being amplified by universal primers, a common problem with this approach, or for removing predator DNA following PCR and prior to cloning/pyrosequencing.

In Chapter 4, earthworm diversity in the diet of four slow worm populations present in different habitats is examined by analysis with general earthworm primers and (for the first time in any terrestrial study) pyrosequencing.

Slow worm diet is more comprehensively explored in Chapter 5, using a suite of species-specific earthworm and pulmonate primers to address hypotheses relating to ontogenetic, seasonal and sex-based differences in predation.

In Chapter 6 the discovery of a parasitic nematode (*Neoxysomatium brevicaudatum*) in UK slow worms is described, along with recordings of their prevalence and intensity, and a comparison is made between determining prevalence by hand and using a PCR approach. This establishes that the molecular method gives comparable results and is far less time-consuming than morphological identification.

In Chapter 7, the molecular approach tested in Chapter 6, using primers specific for *Neoxysomatium brevicaudatum*, is used to analyse parasitic prevalence in slow worms to determine whether there is a sex-bias.

A comparison of the diet of smooth snakes and grass snakes is made in Chapter 8, using a suite of amphibian, reptile, mammal and invertebrate species-specific prey primers, with particular focus on addressing the hypotheses of whether there are ontogenetic, seasonal or sex-based differences in the diet of smooth snakes. The potential for competition (vs. resource partitioning) between smooth snakes and grass snakes is also considered.

Chapter 9 contains the final discussion and conclusions of the PhD.

## 1.9

## References

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## **Chapter 2**

### **Method Development and Preliminary Analyses**

## 2.1 *Introduction*

The purpose of the work detailed in this chapter was to develop methods and establish protocols for subsequent experiments and analyses. Specifically, the aims were to: determine which commercial faecal DNA extraction kits provide the best results; locate/design suitable universal/general primers for dietary analyses of reptiles; identify how quickly faecal DNA degrades and whether there is an optimal mass of faeces for DNA extraction; establish that prey DNA survives digestion in lizards and snakes and is detectable by PCR, along with how soon and for how long prey items become and remain detectable; and determine the practicality of using general primers, cloning and sequencing in dietary analyses of reptiles.

## 2.2 *Study animals and husbandry*

Twelve 8-9 month old sibling checkered garter snakes (*Thamnophis marcianus marcianus*) (six male, six female), purchased from a pet supplier, were kept in separate plastic faunariums (460 x 300 x 170 mm) and 20 slow worms (*Anguis fragilis*) (10 male, 10 female) were kept in separate plastic faunariums (395 x 255 x 155 mm). Due to UK legislation prohibiting the sale of smooth snakes and grass snakes, checkered garter snakes, which have a broad diet and are also a member of the Colubridae family, were selected as a model for them. All animals were kept in a Constant Temperature room maintained at 20°C on L:D cycle of 18:6 h. Paper towels were used as a substrate in each enclosure, and each animal provided with a refugium. They were provided with fresh water every couple of days, and food twice a week for snakes and daily for slow worms. Snakes were fed mouse (*Mus musculus*), chicken (*Gallus gallus*) and earthworm (*Lumbricus terrestris*) on alternating occasions; slow worms were fed on earthworms (*L. terrestris*) and slugs (*Arion* spp. and

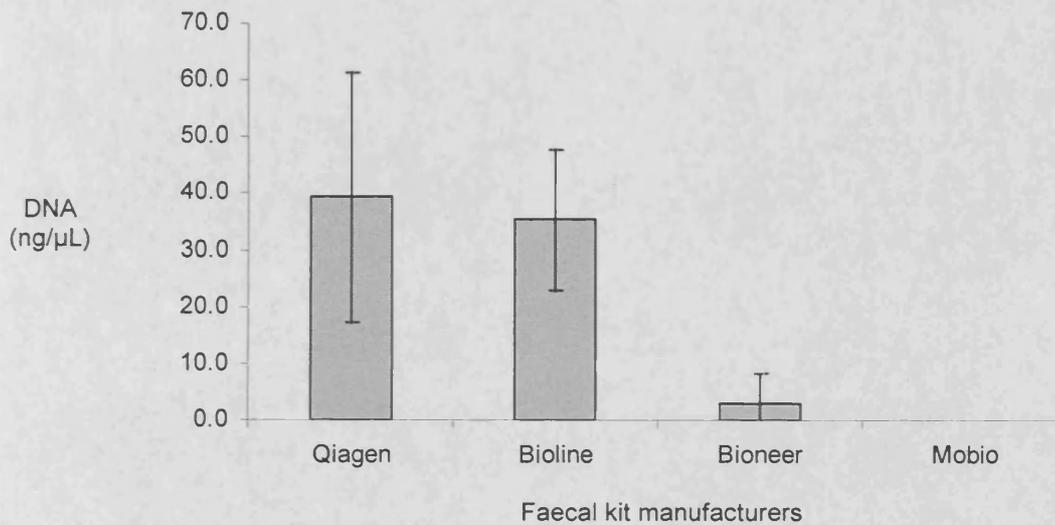
*Deroceras reticulatum*). Faunariums were cleaned weekly, and substrate changed after every defecation.

### 2.3 Faecal extraction kits

Garter snakes were fed a diet of large *L. terrestris*. Enclosures were checked regularly for faeces, which was collected using a sterile spatula, put into 1.5 mL Eppendorf tubes and stored at -20°C. Ten faecal samples were collected from different snakes, with each vortexed thoroughly and divided into four equal parts for extraction by four different commercial faecal extraction kits: the Accuprep® Stool Extraction Kit (Bioneer) (approx. £0.72 each extraction), the Ultraclean® Fecal DNA Kit (Mobio) (approx. £3.57 ea.), the QIAamp® DNA Stool Mini Kit (Qiagen) (approx. £2.64 ea.) and the Fecal PCR kit (Bioline) (approx. £2.80 ea.). Faecal samples were extracted using manufacturer's protocols. Extracted DNA was quantified using the Nanodrop ND-1000 spectrophotometer. Each sample was then screened in PCR with *L. terrestris*-specific primers COII-Lt-F2 (GAATCTATTTTCYACATTTAAGAA) and COII-Lt-R2 (CGGCTATGCTCTYCTAGCAC) (King *et al.* 2010), which amplify a 256 bp fragment of the Cytochrome Oxidase II mitochondrial gene, using 1X Multiplex PCR Master Mix (Qiagen), 0.2 µM of each primer and 5X bovine serum albumin (BSA). PCR cycling conditions were 95 °C for 15 min, and 35 cycles of 94 °C for 30 s, 57 °C for 90 s, 72 °C for 90 s, and a final extension of 72 °C for 10 min.

The Bioneer kit, while the most affordable, produced the lowest yield of DNA, with an average of 3.2 ng/µL compared to averages of 35 ng/µL and 39 ng/µL for the Bioline and Qiagen kits respectively (Fig. 2.1). Quantification of the DNA extracted with the Mobio kit was not possible as the eluate was not pure enough, however when screened with PCR primers specific to the *L. terrestris* prey the snakes had been fed on, 80% of samples ( $n=10$ )

tested positive, which was equal to the Bioneer kit (despite its low DNA yield) and better than the Bioline kit, for which only 70% of samples tested positive (Table 2.1). Using the Qiagen kit, 90% of samples tested positive. Based on this, and the results of the DNA quantification, the Qiagen kit was chosen for all subsequent extractions.



**Figure 2.1.** Quantification of garter snake faecal samples ( $n=10$ ) extracted with different extraction kits: Qiagen, Bioline, Bioneer and Mobio. The Mobio kit didn't produce elute clean enough for DNA quantification by nanodrop. Bars  $\pm$ S.E.

KIT	PROPORTION SUCCESS
Qiagen	0.9
Bioline	0.7
Bioneer	0.8
Mobio	0.8

**Table 2.1.** Proportion of garter snake faecal sample DNA ( $n=10$ ) testing positive for prey (when screened in PCR with *Lumbricus terrestris* primers COII-Lt-F2 and COII-Lt-R2) (King *et al.* 2005) when extracted using kits produced by: Qiagen, Bioline, Bioneer and Mobio.

## 2.4 *Primer development*

### 2.4.1 *Primer design*

All primers designed during the study were done so by eye using BioEdit (version 7.0.4.1) to align homologous sequences of relevant taxa, and NetPrimer (Premier Biosoft International) to test for hairpin structures, self-dimers and cross dimers. Hairpins are formed by an intramolecular interaction within a primer, whereas self-dimers and cross-dimers are formed by intermolecular interactions either between the same sense primers or between the sense and antisense primers respectively. NetPrimer was also used in calculating melting temperatures of primers, as an indication of annealing temperatures, to ensure that they were similar for both primers in a pair. Annealing temperatures too high can result in a low yield, whereas too low can lead to non-specific mis-priming, so primers were designed to anneal within the range of 45 °C and 65 °C. Primers were all designed to be around 18-25 bp, long enough to be specific and short enough to allow primers to bind to the template at an appropriate annealing temperature (i.e. below 65 °C). Having more than four consecutive oligonucleotide or single base repeats in primers was avoided as this can cause mis-priming. Primers were designed to have a GC content (the proportion of G or C bases) of between 40-60%, and the presence of G or C bases within the last five bases within the 3' end of primers, as this encourages specific bonding at the 3' end resulting from the stronger triple bonds between G and C bases. Shorter amplicons are more likely to survive digestion (von Berg *et al.* 2008), making them easier to target, therefore fragments of <300 bp were used.

### 2.4.2 *Mouse- and chicken-specific primers*

Chicken (*G. gallus*) specific primers Galluscytb1\_F (5'-CATGGGGCCAAATATCATTTC-3') and Galluscytb2\_R (5'-GGGGAGGAGGAAGTGTAAAGG-3') (Finden 2004), which target a 158 bp

Cytochrome *b* fragment, were tested for specificity in PCR against extracted DNA of chicken, house mouse (*M. musculus*) and checkered garter snake (*T. marcianus marcianus*) using 1X buffer, 3 mM MgCl<sub>2</sub>, 0.5 mM dNTPs (New England Biolabs), 0.5 μM of each primer, 0.38 U *Taq* polymerase (Invitrogen) and 3 μL/25 μL template DNA. PCR was performed at 94°C for 3 min, 30 cycles of 94 °C for 30 s, 57 °C for 30 s and 72 °C for 45 s, with a final extension at 72 °C for 5 min. Amplification was visualized by gel electrophoresis stained with ethidium bromide. Double-distilled water was included as a negative control to test for contamination. Galluscytb1\_F and Galluscytb2\_R primers were found to cross-amplify with garter snake and specificity could not be achieved through further optimization (including addition of 1-5% Bovine serum albumin (BSA), reduction of primer and of dNTPs, a concentration gradient of MgCl<sub>2</sub> and an annealing temperature gradient between 50°C and 65 °C).

New primers were designed using nucleotide sequences obtained from Genbank (National Centre for Biotechnology Information), using sequences for *Mus musculus* (accession no. NC\_005089), *G. gallus* (accession no. NC\_007236) and *T. marcianus* (accession numbers AF420143 and L33310). Sequences from each species were compared with at least two other sequences of the same species from Genbank to ensure there was no appreciable intraspecific variation. As there was slight variation between sequences for *T. marcianus* two sequences were used to aid primer design. Sequences were aligned using BioEdit (version 7.0.4.1). In total, six sense and six antisense primers were designed for mouse (Table 2.2a) creating thirteen possible combinations which produce fragments between 90 and 350 bp. Ten of these combinations were tested / optimized. Ten sense and eleven antisense primers were designed for chick (Table 2.2b), leading to 44 possible combinations for fragments between 90 and 350bp, of which 32 were tested / optimized.

	<b>SENSE</b>	<b>SEQUENCE 5' TO 3'</b>	<b>ANTIENSE</b>	<b>SEQUENCE 5' TO 3'</b>
a)	MUS-J-14701	TGACAAACATACGAAAAACACACCCAT	MUS-N-14849	GAAAAGACCTGTAATGATTTGGACT
	MUS-J-14705	AAACATACGAAAAACACACCCAT	MUS-N-14905	ATGTGTGTTACTGATGAAAAGCTGTTAT
	MUS-J-14761	TACCTGCCCCATCCAACATTTTCAT	MUS-N-15039	AATGTTTCAGGTTTCTATAAATGTA
	MUS-J-14850	CATACACTACACATCAGATACAATAAC	MUS-N-15294	AGAGGAGGTGAACGATTGCTAGG
	MUS-J-15149	ATCAGCCATCCCATATATTGGAAC	MUS-N-15346	TCTGCATCTGAGTTAATCCTGTT
	MUS-J-15313	CAAACCTAACCCAACAGGATTAAC	MUS-N-15419	TATGAGAATTAAGAAATATGATTAGGATA
b)	GAL-J-14970	CAGCCCCATCCAACATCTCTG	GAL-N-15138	AGTCAGCCGTATTGTACGTTT
	GAL-J-14971	AGCCCCATCCAACATCTCT	GAL-N-15246	TTTCAGGTTTCCTTGAGAGG
	GAL-J-15008	TCCCTATTAGCAGTCTGCCTCATGA	GAL-N-15250	TGTGTTTCAGGTTTCCTTGAGAGG
	GAL-J-15012	TATTAGCAGTCTGCCTCATGAC	GAL-N-15269	TGAGGAGGAGGATTACTCCTG
	GAL-J-15064	GCACTACACAGCAGACATCCCT	GAL-N-15276	ATTAGTGTGAGGAGGATTACTCCTG
	GAL-J-15163	CGCCTCATTCTTCTCATCTGTA	GAL-N-15289	AAAGGCGGTGGCTATTAGTGTGAGG
	GAL-J-15373	GGACACACCCTAGTAGAGTGAGCC	GAL-N-15561	GGAATTTTGTGAGAGTCGGTGAG
	GAL-J-15596	GGCTTAACTCTCATACTCACCCC	GAL-N-15653	GGAGGTTGGGGGAGAATAGGG
	GAL-J-15613	CACCCATTCTTAACACTAGCCC	GAL-N-15810	AAGAGGATGAGGACTGAGGCTGC
	GAL-J-15771	GAGGTGTACTAGCCCTAGCAGCC	GAL-N-15868	GGAGAGTGGTCGGAAGTTATTG
			GAL-N-16033	TATTTTGTCTTCTAGTGTCCGATTG

**Table 2.2.** a) Cytochrome *b* primers designed and 10 combinations tested for mouse-specificity\* in PCR; b) Cytochrome *b* primers designed and 32 combinations tested for chicken-specificity\*.

\* specificity only in relation to mouse (*Mus musculus*), chick (*Gallus galus*), checkered garter snake (*Thamnophis marcianus marcianus*) and earthworm (*Lumbricus terrestris*).

The DNeasy® Tissue Kit (Qiagen) was used for extraction of DNA from sloughed garter snake (*T. marcianus*) skin, chicken (*G. gallus*) tissue and mouse (*M. musculus*) tail (rodent tail protocol). Extracts were tested with general primers MCB398 and MCB869 (Kocher *et al.* 1989, using author's conditions) which flank a 471 bp region of Cytochrome *b*, to confirm successful DNA extraction and the absence of PCR inhibitors which would cause false negative results. A temperature gradient PCR was performed for each primer pair, with annealing temperatures ranging from 48-68 °C, to determine the highest temperature a primer pair amplified the target. Target-specificity testing was accomplished by PCR with mouse or chicken specific primers on tissue DNA extracts, with an initial denaturation at 94 °C for 3 min, 35 cycles of 94 °C for 30 s, the optimum annealing temperature for the primer

SPECIES	PRIMERS	GENE	DETAILS
Earthworm (group)	185F / 14233R *	12S	Ch 4, 5
<i>Allolobophora chlorotica</i>	various <sup>a</sup>	COI	Ch 5
<i>Aporrectodea caliginosa</i>	16S-Ac-F1 / WORM-16S-R1 <sup>a</sup>	16S	Ch 5
<i>A. longa</i>	COI-AI-F2 / COI-AI-R2 <sup>a</sup>	COI	Ch 5
<i>A. rosea</i>	various <sup>a</sup>	COI	Ch 5
<i>Lumbricus castaneus</i>	COI-Lc-F2 / COI-Lc-R2 <sup>a</sup>	COI	Ch 5
<i>L. rubellus</i>	COII-Lr-F3 / COII-Lr-R2 <sup>a</sup>	COII	Ch 5
<i>L. terrestris</i>	COII-Lt-F2 / COII-Lt-R2 <sup>a</sup>	COII	Ch 5
Arion spp.	Ai1F / AR2R *	12S	Ch 5
<i>Deroceras reticulatum</i>	Dr11F / DRF29RC *	12S	Ch 5
<i>Sorex araneus</i>	SA520 / SA628 <sup>b</sup>	cyt b	Ch 8
<i>S. minutus</i>	SM421 / SM544 <sup>b</sup>	cyt b	Ch 8
<i>Clethrionomys glareolus</i>	CG95 / CG266 <sup>b</sup>	cyt b	Ch 8
<i>Anguis fragilis</i>	SW-J-1495 (ACAAATCATAAAGATATTGGAACCCTC)	COI	Ch 8
	SW-N-1730 (CCAATTATTAGTGGGACGAG)	COI	Ch 8
	SW-N-110 (GGCTGGCTTAACTCTGCG)	COI	Ch 8
<i>Rana temporaria</i>	RANA-F (TACAGCCGATACCTCCCTC)	cyt b	Ch 8
	RANA-R (TTCATGTCTCTTTGTAGAGG)	cyt b	Ch 8
<i>Lissotriton vulgaris</i>	TRIT-F (GACTCGTACGAAACATCCA)	cyt b	Ch 8
	TRIT-R (CGCCTATATATGGAATAGCGG)	cyt b	Ch 8
<i>Lacerta vivipara</i>	CL-216-R (CCCGAACCCACCAATTATTAC)	COI	Ch 8
<i>Neoxysomatium brevicaudatum</i>	COI-J-1764 (TCTTAGATTTTGACTTTTGCCTACAG)	COI	Ch 7
	COI-N-1938 (AGAACTAACACCAGCACAATGTAATC)	COI	Ch 7

**Table 2.3.** Species- and group-specific primers used in the study, described in the relevant chapters. \* Harper *et al.* 2005; <sup>a</sup> King *et al.* 2010; <sup>b</sup> Moran, Turner & O'Reilly 2008.

pair for 45 s and 72 °C for 1 min, and a final extension at 72 °C for 5 min. Double distilled water in place of DNA as a negative control. Amplification success was visualized by gel electrophoresis and ethidium bromide staining. When mouse-specific and chicken-specific primers repeatedly failed to amplify any product from any of the DNA extracts they were screened for a second time with general primers MCB398 and MCB869 with no success. This apparent inhibition of PCRs was remediated by replacing dNTP (New England Biolabs) with dNTP (Invitrogen). Recurring contamination issues (as identified by the presence of bands in the negative control) were overcome by systematically replacing all reagents: PCR water, 10X buffer, MgCl<sub>2</sub>, primers, and *Taq* polymerase (Invitrogen). Specificity could not be achieved for any chicken-specific primer pairs, but mouse-specific primers Mus-J-14701

and Mus-N-14905, which produce a 206 bp fragment, were specific at an annealing temperature of 55.5 °C.

Species-specific primers from the literature were optimized, or new ones designed, for the species shown in Table 2.3, described in the relevant chapters.

### 2.4.3 *Universal primers*

In order to study unexpected components of the diet of wild snakes and slow worms, an approach was taken using universal primers to amplify all prey DNA in faeces, followed by cloning and sequencing to allow species identities to be determined.

#### 2.4.3.1 *18S primers*

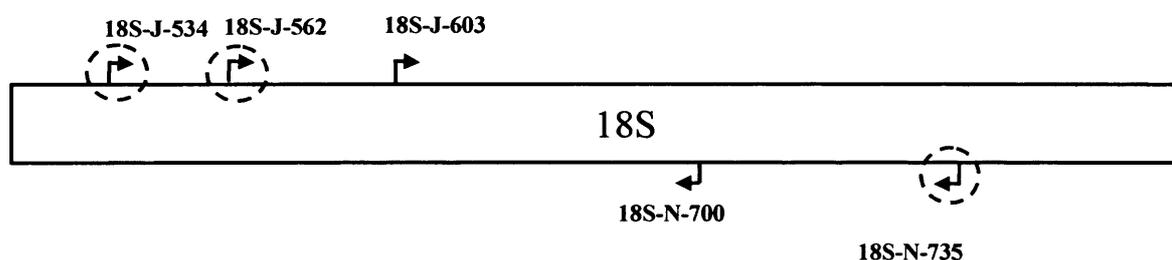
Primers were designed for nuclear 18S rRNA, known to contain extremely conserved regions (Hillis and Dixon 1991). Primers are shown in Table 2.4; and Table 2.5 shows primers 18S-J-534 and 18S-N-735 along with comparative sequences for sixteen species from nine orders. An additional forward primer, 18S-J-562, was also designed. Sequences were obtained from GenBank and aligned on BioEdit using the ClusterW algorithm, and primers were tested using Net Primer for potential dimers, cross dimers, hairpins and compatible melting temperatures. The two combinations of primers were tested against 28 species from 15 orders in PCR using 1X buffer, 2 mM MgCl<sub>2</sub>, 0.5mM dNTP (Invitrogen),

PRIMER	SEQUENCE 5' TO 3'
18S-J-534	TAATTCCAGCTCCAATAGC
18S-J-562	AGTTGTTGCGGTAAAAAG
18S-N-735	CACTCTAATTTTTTCAAAGTAAAC
18S-J-603-SW	TCGTAGTTGGATCTTGGGATCG
18S-N-669-SW	ATGGGACAGGCGGTAGC

**Table 2.4.** Universal 18S primer sequences (18s-J-534, 18S-J-562 and 18S-N-735) and slow worm-specific primers for use in conjunction with universal primers (18S-J-603-SW and 18S-N-669-SW)

0.5  $\mu\text{M}$  of each primer, 0.38 U *Taq* polymerase (Invitrogen) with 2  $\mu\text{L}$ /25  $\mu\text{L}$  of target DNA. A PCR cycle was used comprising an initial denaturation at 94  $^{\circ}\text{C}$  for 3 min, 35 cycles of 94  $^{\circ}\text{C}$  for 30 s, 46  $^{\circ}\text{C}$  for 45 s and 72  $^{\circ}\text{C}$  for 1 min, and a final extension at 72  $^{\circ}\text{C}$  for 5 min. Both amplified 86% of taxa tested (although not the same species, Table 2.5). Primers 18S-J-534 and 18S-N-735 were used to amplify slow worm DNA, and were cleaned using ExoSAP in the following reaction: 10  $\mu\text{L}$  of each PCR product, 0.25  $\mu\text{L}$  Exonuclease I, 0.5  $\mu\text{L}$  SAP (shrimp alkaline phosphatase) and incubated for 45 min at 37 $^{\circ}\text{C}$  and 15 min at 80 $^{\circ}\text{C}$ . Cleaned product was then used in sequencing PCR using a Big Dye<sup>TM</sup> terminator sequencing kit (Promega, Madison, WI, USA).

Species-specific primers were designed for slow worm, in the variable loop region of the 18S gene, to use in conjunction with the general ones, using the PCR conditions described above (Fig. 2.2, Table 2.4). Tested against the same range of 28 species described above, these were found to be slow worm-specific primers at all temperatures (above 50 $^{\circ}\text{C}$ ). These were to be modified as a slow worm-specific blocking primer for removing dominant predator (slow worm) DNA during amplification of prey with universal primers (see Chapter 3). However, after detailed searching of homologous 18S sequences on Genbank, it was



**Figure 2.2.** Diagram of the 18S nuclear rRNA gene showing positions of general 18S primers (dashed circles) and species-specific primers designed for slow worm (*Anguis fragilis*).

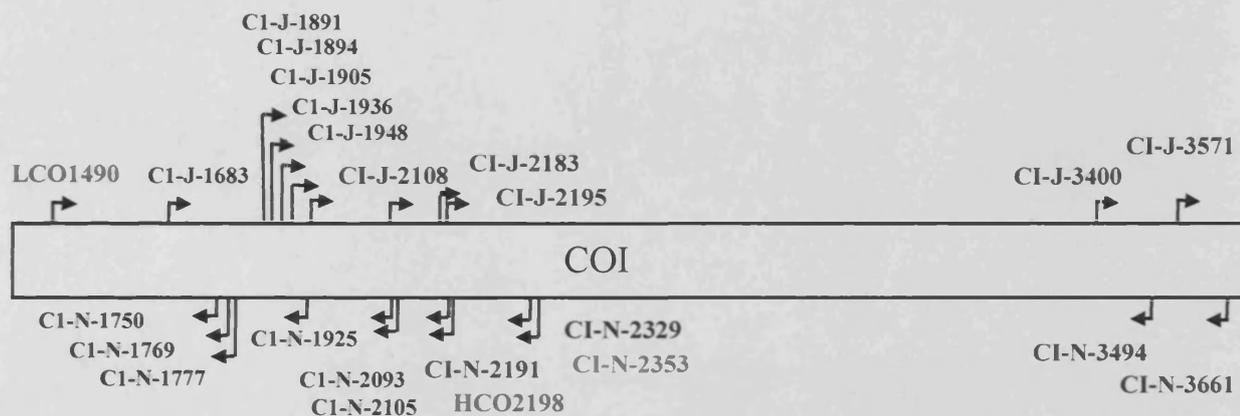
	Species	Order, Family	18S-J-534 / 18S-N-735	18S-J-563 / 18S-N-735
			50 °C	50 °C
Mite	<i>Acaridae</i> sp.	(Acarina: Acaridae)	√	-
Spider	<i>Erigone dentipalpis</i>	(Araneae: Linyphiidae)	√	√
Ground beetle	<i>Notiophilus biguttatus</i>	(Coleoptera: Carabidae)	√	√
Leaf beetle	<i>Oulema melangus</i>	(Coleoptera: Chrysomelidae)	√	√
Lady beetle	<i>Adalia bipunctata</i>	(Coleoptera: Coccinellidae)	√	√
Rove beetle	<i>Tachyporus obtusus</i>	(Coleoptera: Staphylinidae)	-	√
Earwig	<i>Forficula</i> sp.	(Dermaptera: Forficulidae)	√	√
Hoverfly	<i>Scaeva pyrausti</i>	(Diptera: Syrphidae)	√	√
Hoverfly	<i>Metasyrphus luniger</i>	(Diptera: Syrphidae)	√	-
Crane fly	<i>Tipulidae</i> sp.	(Diptera: Tipulidae)	-	-
Earthworm	<i>Lumbricus terrestris</i>	(Haplotaxida: Lumbricidae)	√	√
Earthworm	<i>Aporrectodea caliginosa</i>	(Haplotaxida: Lumbricidae)	√	√
Earthworm	<i>Aporrectodea longa</i>	(Haplotaxida: Lumbricidae)	√	√
Earthworm	<i>Lumbricus rubellus</i>	(Haplotaxida: Lumbricidae)	√	√
Aphid	<i>Aphis fabae</i>	(Hemiptera: Aphididae)	-	-
Spittlebug	<i>Formicidae</i> sp.	(Hymenoptera: Formicidae)	√	√
Moth	<i>Galleria mellonella</i>	(Lepidoptera: Pyralidae)	-	√
Slug	<i>Deroceras reticulatum</i>	(Pulmonata: Agriolimacidae)	√	√
Slug	<i>Arion intermedius</i>	(Pulmonata: Arionidae)	√	√
Slug	<i>Arion owenii</i>	(Pulmonata: Arionidae)	√	√
Slug	<i>Arion distinctus</i>	(Pulmonata: Arionidae)	√	√
Slug	<i>Arion hortensis</i>	(Pulmonata: Arionidae)	√	√
Slug	<i>Limax flavus</i>	(Pulmonata: Limacidae)	√	√
Thrip	<i>Thripidae</i> sp.	(Thysanoptera: Thripidae)	√	√
Chicken	<i>Gallus gallus</i>	(Galliformes: Phasianidae)	√	√
Mouse	<i>Mus musculus</i>	(Rodentia: Muridae)	√	√
Slow worm	<i>Anguis fragilis</i>	(Sauria: Anguidae)	√	√
Smooth snake	<i>Coronella austriaca</i>	(Squamata: Colubridae)	√	√
			<b>86%</b>	<b>86%</b>

**Table 2.5.** 18S universal primers tested against a range of taxa (√ indicates amplification success).

deemed that this gene would not be as informative as COI due to the smaller number of taxa represented in the database, which would result in poorer resolution of prey identity.

## 3.3.2 COI

Universal COI primers were designed and used in combination with those designed by Simon *et al.* (1994) and Folmer *et al.* (1994). In total, 12 forward and 12 reverse primers were tested in 33 (of a possible 35 combinations) which produce fragments between 80-310 bp (Table 2.6 and Fig. 2.3). Primers were tested for amplification success against DNA from



**Figure 2.3.** Diagram of the Cytochrome Oxidase I (COI) mitochondrial gene showing positions of general primers tested, including self-designed primers in conjunction with previously published primers (Simon *et al.* 1994 (blue); Folmer *et al.* 1994 (red); Simon *et al.* 2006 (green)).

	C1-N-1750	C1-N-1769	C1-N-1777	C1-N-1925	C1-N-2093	C1-N-2105	C1-N-2191 <sup>†</sup>	HCO-2198*	C1-N-2329 <sup>†</sup>	C1-N-2353 <sup>α</sup>	C1-N-3494 <sup>†</sup>	C1-N-3661 <sup>†</sup>
LCO1490 <sup>†</sup>	260	279	287									
C1-J-1683		86	94	242								
C1-J-1891					202	214	300					
C1-J-1894					199	211	297	304				
C1-J-1905					188	200	286	293				
C1-J-1936					157	169	255	262				
C1-J-1948					145	157	243	250				
C1-J-2108 <sup>†</sup>								90	221	245		
C1-J-2183 <sup>†</sup>									146	170		
C1-J-2195 <sup>†</sup>									134	158		
C1-J-3400 <sup>†</sup>											94	261
C1-J-3571 <sup>†</sup>												90

**Table 2.6.** COI universal primer combinations which produce fragments of between 80 and 350 bp (\* primers in Folmer *et al.* 1994, <sup>†</sup> primers in Simon *et al.* 1994, <sup>α</sup> primer in Simon *et al.* 2006).

a range of species from 22 different families. PCR conditions were as follows: 1X buffer, 2 mM MgCl<sub>2</sub>, 0.5 mM dNTP (Invitrogen), 0.5 μM of each primer, 0.38 U *Taq* polymerase (Invitrogen) and 2 μL/ 25 μL of DNA with an initial denaturation at 94 °C for 3 min, 35 cycles of 94 °C for 30 s, 48 °C for 30 s and 72 °C for 45 s, and a final extension at 72 °C for 5 min. Amplification was visualized by gel electrophoresis stained with ethidium bromide. Double-distilled water was included as a negative control to test for contamination. Most primer combinations either amplified limited or no taxa (Table 2.7); however, primer pair LCO1490 and C1-N-1777 which amplify a 287 bp amplicon, amplified all taxa tested and was chosen for subsequent analysis (Chapters 3).

#### 2.4 *Detection of prey DNA in faeces*

Garter snakes and slow worms were maintained on a diet of *Lumbricus terrestris* earthworms. Enclosures were checked on an hourly basis so that faeces could be guaranteed to be no more than an hour old. Ten fresh faecal samples were collected from snakes and ten from slow worms. Samples were weighed and DNA extracted from each of the whole samples using the QIAamp® DNA Stool Mini Kit. Extractions were quantified using the Nanodrop ND-1000 spectrophotometer. There was no correlation between initial faecal weight and DNA concentration (Fig. 2.4a,b) for either garter snakes (Regression: DF=1,18, F=0.47,  $p=0.50$ ) or slow worms (Regression: DF=1,16, F=1.97,  $p=0.18$ ).

In a second experiment, fresh faecal samples from garter snakes were collected and pooled together and divided out into eight 300mg samples on a Petri dish and left in the CT room at 20°C. At various time points (0h, 6h, 12h, 24h, 48h, 72h, 120h, and 168h) each of the eight samples were extracted using the QIAamp® DNA Stool Mini Kit. This was repeated ten times. This was also done using slow worm faeces, although 72h and 168h

Species	Order, Family	LCO1490 / C1-N-1750 = 260 bp		LCO1490 / C1-N-1769 = 279 bp		LCO1490 / C1-N-1777 = 287 bp		C1-J-1683 / C1-N-1769 = 86 bp	
		50 °C	55 °C	50 °C	55 °C	50 °C	55 °C	50 °C	55 °C
Mite	Acaridae sp. (Acarina: Acaridae)	-	-	√	-	√	√	-	-
Spider	<i>Erigone dentipalpis</i> (Araneae: Linyphiidae)	√	√	-	-	√	-	-	-
Ground beetle	<i>Notiophilus biguttatus</i> (Coleoptera: Carabidae)	√	√	-	-	√	√	-	-
Leaf beetle	<i>Oulema melangus</i> (Coleoptera: Chrysomelidae)	√	√	√	-	√	√	-	-
Lady beetle	<i>Adalia bipunctata</i> (Coleoptera: Coccinellidae)	√	√	-	-	√	-	-	-
Rove beetle	<i>Tachyporus obtusus</i> (Coleoptera: Staphylinidae)	-	√	-	-	√	√	-	-
Earwig	<i>Forficula</i> sp. (Dermaptera: Forficulidae)	-	√	-	-	√	√	-	-
Hoverfly	<i>Scaeva pyrausti</i> (Diptera: Syrphidae)	√	√	√	√	√	√	-	-
Hoverfly	<i>Metasyrphus luniger</i> (Diptera: Syrphidae)	√	-	-	-	√	-	-	-
Cranefly	Tipulidae sp. (Diptera: Tipulidae)	-	-	√	-	√	√	-	-
Earthworm	<i>Lumbricus terrestris</i> (Haplotaxida: Lumbricidae)	√	√	-	-	√	√	-	-
Earthworm	<i>Aporrectodea caliginosa</i> (Haplotaxida: Lumbricidae)	-	√	-	-	√	-	-	-
Earthworm	<i>Aporrectodea longa</i> (Haplotaxida: Lumbricidae)	√	√	-	-	√	√	-	-
Earthworm	<i>Lumbricus rubellus</i> (Haplotaxida: Lumbricidae)	√	√	√	-	√	√	-	-
Earthworm	<i>Aporrectodea rosae</i> (Haplotaxida: Lumbricidae)					√	√		
Earthworm	<i>Aporrectodea chlorotica</i> (Haplotaxida: Lumbricidae)					√	√		
Aphid	<i>Aphis fabae</i> (Hemiptera: Aphididae)	-	-	√	-	√	√	-	-
Aphid	<i>Rhopalosiphum padi</i> (Hemiptera: Aphididae)					√	√		
Spittlebug	Formicidae sp. (Hymenoptera: Formicidae)	-	-	-	-	√	√	-	-
Moth	<i>Galleria mellonella</i> (Lepidoptera: Pyralidae)	-	-	-	-	√	√	-	-
Slug	<i>Deroceras reticulatum</i> (Pulmonata: Agriolimacidae)	-	-	√	√	√	√	-	-
Slug	<i>Arion intermedius</i> (Pulmonata: Arionidae)	-	-	√	√	√	-	-	-
Slug	<i>Arion owenii</i> (Pulmonata: Arionidae)	-	-	√	√	√	-	-	-
Slug	<i>Arion distinctus</i> (Pulmonata: Arionidae)	-	-	-	-	√	√	-	-
Slug	<i>Arion hortensis</i> (Pulmonata: Arionidae)	√	-	√	√	√	√	-	-
Slug	<i>Limax flavus</i> (Pulmonata: Limacidae)	-	-	-	-	√	√	-	-
Thrip	Thripidae sp. (Thysanoptera: Thripidae)	-	-	-	-	√	√	-	-
Chicken	<i>Gallus gallus</i> (Galliformes: Phasianidae)					√	-		
Mouse	<i>Mus musculus</i> (Rodentia: Muridae)					√	√		
Slow worm	<i>Anguis fragilis</i> (Sauria: Anguidae)	-	-	√	-	√	√	-	-
Smooth snake	<i>Coronella austriaca</i> (Squamata: Colubridae)	-	-	-	-	√	√	-	-
Garter snake	<i>Thamnophis marcianus</i> (Squamata: Natricidae)					√	-		
		<b>39%</b>	<b>4%</b>	<b>42%</b>	<b>19%</b>	<b>100%</b>	<b>75%</b>	<b>0%</b>	<b>0%</b>

Species	Order, Family	C1-J-1683 / C1-N-1777 = 94 bp		C1-J-1683 / C1-N-1925 = 242 bp		C1-J-1894 / HCO2198 = 304 bp		C1-J-1905 / HCO2198 = 293 bp	
		50 °C	55 °C	50 °C	55 °C	50 °C	55 °C	50 °C	55 °C
Mite	Acaridae sp. (Acarina: Acaridae)	-	-	-	-	-	-	-	-
Spider	<i>Erigone dentipalpis</i> (Araneae: Linyphiidae)	-	-	-	-	-	-	-	-
Ground beetle	<i>Notiophilus biguttatus</i> (Coleoptera: Carabidae)	-	-	-	-	-	-	-	-
Leaf beetle	<i>Oulema melangus</i> (Coleoptera: Chrysomelidae)	-	-	-	-	-	-	-	-
Lady beetle	<i>Adalia bipunctata</i> (Coleoptera: Coccinellidae)	-	-	-	-	-	-	-	-
Rove beetle	<i>Tachyporus obtusus</i> (Coleoptera: Staphylinidae)	-	-	-	-	-	-	-	-
Earwig	<i>Forficula</i> sp. (Dermaptera: Forficulidae)	-	-	-	-	-	-	-	-
Hoverfly	<i>Scaeva pyrastii</i> (Diptera: Syrphidae)	-	-	√	-	-	-	-	-
Hoverfly	<i>Metasyrphus luniger</i> (Diptera: Syrphidae)	-	-	-	-	-	-	-	-
Crane fly	Tipulidae sp. (Diptera: Tipulidae)	-	-	-	-	-	-	-	-
Earthworm	<i>Lumbricus terrestris</i> (Haplotaxida: Lumbricidae)	-	-	-	-	-	-	-	-
Earthworm	<i>Aporrectodea caliginosa</i> (Haplotaxida: Lumbricidae)	-	-	-	-	-	-	-	-
Earthworm	<i>Aporrectodea longa</i> (Haplotaxida: Lumbricidae)	-	-	-	-	-	-	-	-
Earthworm	<i>Lumbricus rubellus</i> (Haplotaxida: Lumbricidae)	-	-	-	-	-	-	-	-
Earthworm	<i>Aporrectodea rosae</i> (Haplotaxida: Lumbricidae)								
Earthworm	<i>Aporrectodea chlorotica</i> (Haplotaxida: Lumbricidae)								
Aphid	<i>Aphis fabae</i> (Hemiptera: Aphididae)	-	-	-	-	-	-	-	-
Aphid	<i>Rhopalosiphum padi</i> (Hemiptera: Aphididae)								
Spittlebug	Formicidae sp. (Hymenoptera: Formicidae)	-	-	-	-	-	-	-	-
Moth	<i>Galleria mellonella</i> (Lepidoptera: Pyralidae)	-	-	-	-	-	-	-	-
Slug	<i>Deroceras reticulatum</i> (Pulmonata: Agriolimacidae)	-	-	-	-	-	-	-	-
Slug	<i>Arion intermedius</i> (Pulmonata: Arionidae)	-	-	√	-	-	-	-	-
Slug	<i>Arion owenii</i> (Pulmonata: Arionidae)	-	-	√	-	-	-	-	-
Slug	<i>Arion distinctus</i> (Pulmonata: Arionidae)	-	-	-	-	-	-	-	-
Slug	<i>Arion hortensis</i> (Pulmonata: Arionidae)	-	-	√	-	-	-	-	-
Slug	<i>Limax flavus</i> (Pulmonata: Limacidae)	-	-	-	-	-	-	-	-
Thrip	Thripidae sp. (Thysanoptera: Thripidae)	-	-	-	-	-	-	-	-
Chicken	<i>Gallus gallus</i> (Galliformes: Phasianidae)								
Mouse	<i>Mus musculus</i> (Rodentia: Muridae)								
Slow worm	<i>Anguis fragilis</i> (Sauria: Anguidae)	-	-	-	-	-	-	-	-
Smooth snake	<i>Coronella austriaca</i> (Squamata: Colubridae)	-	-	-	-	-	-	-	-
Garter snake	<i>Thamnophis marcianus</i> (Squamata: Natricidae)								
		0%	0%	15%	0%	0%	0%	0%	0%

Species	Order, Family	C1-J-1936 / C1-N-2093 = 157 bp		C1-J-1936 / C1-N-2105 = 169 bp		C1-J-1948 / C1-N-2191 = 243 bp		C1-J-1948 / HCO2198 = 250 bp	
		50 °C	55 °C	50 °C	55 °C	50 °C	55 °C	50 °C	55 °C
Mite	Acaridae sp. (Acarina: Acaridae)	-	-	-	-	-	-	-	-
Spider	<i>Erigone dentipalpis</i> (Araneae: Linyphiidae)	-	-	-	-	-	-	-	-
Ground beetle	<i>Notiophilus biguttatus</i> (Coleoptera: Carabidae)	-	-	-	-	√	√	-	-
Leaf beetle	<i>Oulema melanopus</i> (Coleoptera: Chrysomelidae)	-	-	-	-	-	-	-	-
Lady beetle	<i>Adalia bipunctata</i> (Coleoptera: Coccinellidae)	-	-	-	-	-	-	-	-
Rove beetle	<i>Tachyporus obtusus</i> (Coleoptera: Staphylinidae)	-	-	-	-	-	-	-	-
Earwig	<i>Forficula</i> sp. (Dermaptera: Forficulidae)	-	-	-	-	-	-	-	-
Hoverfly	<i>Scaeva pyrastii</i> (Diptera: Syrphidae)	-	-	-	-	-	-	-	-
Hoverfly	<i>Metasyrphus luniger</i> (Diptera: Syrphidae)	-	-	-	-	-	-	-	-
Cranefly	Tipulidae sp. (Diptera: Tipulidae)	-	-	-	-	√	√	-	-
Earthworm	<i>Lumbricus terrestris</i> (Haplotaxida: Lumbricidae)	-	-	-	-	√	√	-	-
Earthworm	<i>Aporrectodea caliginosa</i> (Haplotaxida: Lumbricidae)	-	-	-	-	-	-	-	-
Earthworm	<i>Aporrectodea longa</i> (Haplotaxida: Lumbricidae)	-	-	-	-	-	-	-	-
Earthworm	<i>Lumbricus rubellus</i> (Haplotaxida: Lumbricidae)	-	-	-	-	-	-	-	-
Earthworm	<i>Aporrectodea rosae</i> (Haplotaxida: Lumbricidae)	-	-	-	-	-	-	-	-
Earthworm	<i>Aporrectodea chlorotica</i> (Haplotaxida: Lumbricidae)	-	-	-	-	-	-	-	-
Aphid	<i>Aphis fabae</i> (Hemiptera: Aphididae)	-	-	-	-	-	-	-	-
Aphid	<i>Rhopalosiphum padi</i> (Hemiptera: Aphididae)	-	-	√	√	-	-	-	-
Spittlebug	Formicidae sp. (Hymenoptera: Formicidae)	-	-	-	-	-	-	-	-
Moth	<i>Galleria mellonella</i> (Lepidoptera: Pyralidae)	-	-	-	-	-	-	-	-
Slug	<i>Deroceras reticulatum</i> (Pulmonata: Agriolimacidae)	-	-	-	-	√	√	-	-
Slug	<i>Arion intermedius</i> (Pulmonata: Arionidae)	-	-	-	-	√	√	-	-
Slug	<i>Arion owenii</i> (Pulmonata: Arionidae)	-	-	-	-	√	√	-	-
Slug	<i>Arion distinctus</i> (Pulmonata: Arionidae)	-	-	-	-	√	√	-	-
Slug	<i>Arion hortensis</i> (Pulmonata: Arionidae)	-	-	-	-	√	√	-	-
Slug	<i>Limax flavus</i> (Pulmonata: Limacidae)	-	-	-	-	√	√	-	-
Thrip	Thripidae sp. (Thysanoptera: Thripidae)	-	-	-	-	-	-	-	-
Chicken	<i>Gallus gallus</i> (Galliformes: Phasianidae)	-	-	√	-	-	-	-	-
Mouse	<i>Mus musculus</i> (Rodentia: Muridae)	-	-	√	-	-	-	-	-
Slow worm	<i>Anguis fragilis</i> (Sauria: Anguidae)	-	-	-	-	√	-	-	-
Smooth snake	<i>Coronella austriaca</i> (Squamata: Colubridae)	-	-	-	-	-	-	-	-
Garter snake	<i>Thamnophis marciianus</i> (Squamata: Natricidae)	-	-	-	-	-	-	-	-
		0%	0%	12%	4%	38%	34%	0%	0%

Species	Order, Family	= 90 bp		= 221 bp		= 146 bp		= 170 bp	
		50 °C	55 °C	50 °C	55 °C	50 °C	55 °C	50 °C	55 °C
Mite	Acaridae sp. (Acarina: Acaridae)			-	-	-	-	-	
Spider	<i>Erigone dentipalpis</i> (Araneae: Linyphiidae)	√	√	-	-	-	-	√	√
Ground beetle	<i>Notiophilus biguttatus</i> (Coleoptera: Carabidae)	√	√	-	-	-	√	√	√
Leaf beetle	<i>Oulema melangus</i> (Coleoptera: Chrysomelidae)	√	√	-	-	√	√	√	√
Lady beetle	<i>Adalia bipunctata</i> (Coleoptera: Coccinellidae)	√	√	√	-	√	√	√	√
Rove beetle	<i>Tachyporus obtusus</i> (Coleoptera: Staphylinidae)	√	√	-	-	-	-	√	√
Earwig	<i>Forficula</i> sp. (Dermaptera: Forficulidae)	√	√	-	-	√	√	-	-
Hoverfly	<i>Scaeva pyrausti</i> (Diptera: Syrphidae)	√	√	-	-	√	√	√	√
Hoverfly	<i>Metasyrphus luniger</i> (Diptera: Syrphidae)			-	-	-	√	√	
Cranefly	Tipulidae sp. (Diptera: Tipulidae)	√	√	-	-	-	√	√	√
Earthworm	<i>Lumbricus terrestris</i> (Haplotaxida: Lumbricidae)	√	√	-	-	-	-	-	√
Earthworm	<i>Aporrectodea caliginosa</i> (Haplotaxida: Lumbricidae)	-	√	-	-	-	-	-	√
Earthworm	<i>Aporrectodea longa</i> (Haplotaxida: Lumbricidae)	√	√	-	-	-	-	-	√
Earthworm	<i>Lumbricus rubellus</i> (Haplotaxida: Lumbricidae)	√	√	-	-	-	-	-	√
Earthworm	<i>Aporrectodea rosae</i> (Haplotaxida: Lumbricidae)	√	√	-	-				√
Earthworm	<i>Aporrectodea chlorotica</i> (Haplotaxida: Lumbricidae)	√	√	-	-				√
Aphid	<i>Aphis fabae</i> (Hemiptera: Aphididae)	√	√	-	-	-	√	√	√
Aphid	<i>Rhopalosiphum padi</i> (Hemiptera: Aphididae)	√	√	-	-				√
Spittlebug	Formicidae sp. (Hymenoptera: Formicidae)			-	-	√	√	√	
Moth	<i>Galleria mellonella</i> (Lepidoptera: Pyralidae)			-	-	-	-	-	
Slug	<i>Deroceras reticulatum</i> (Pulmonata: Agriolimacidae)	√	√	-	-	-	-	√	√
Slug	<i>Arion intermedius</i> (Pulmonata: Arionidae)	√	√	-	-	√	√	√	√
Slug	<i>Arion owenii</i> (Pulmonata: Arionidae)	√	√	-	-	√	√	√	√
Slug	<i>Arion distinctus</i> (Pulmonata: Arionidae)	√	√	-	-	-	-	√	√
Slug	<i>Arion hortensis</i> (Pulmonata: Arionidae)	-	√	-	-	-	-	-	√
Slug	<i>Limax flavus</i> (Pulmonata: Limacidae)	√	√	-	-	√	√	√	-
Thrip	Thripidae sp. (Thysanoptera: Thripidae)	√	√	-	-	√	√	√	√
Chicken	<i>Gallus gallus</i> (Galliformes: Phasianidae)	-	-	-	-				√
Mouse	<i>Mus musculus</i> (Rodentia: Muridae)	-	-	-	-				-
Slow worm	<i>Anguis fragilis</i> (Sauria: Anguidae)	-	-	-	-	-	-	-	-
Smooth snake	<i>Coronella austriaca</i> (Squamata: Colubridae)	-	-	-	-	-	-	-	√
Garter snake	<i>Thamnophis marciianus</i> (Squamata: Natricidae)	-	-	-	-	-	-	-	√
		75%	82%	3%	0%	33%	48%	59%	89%

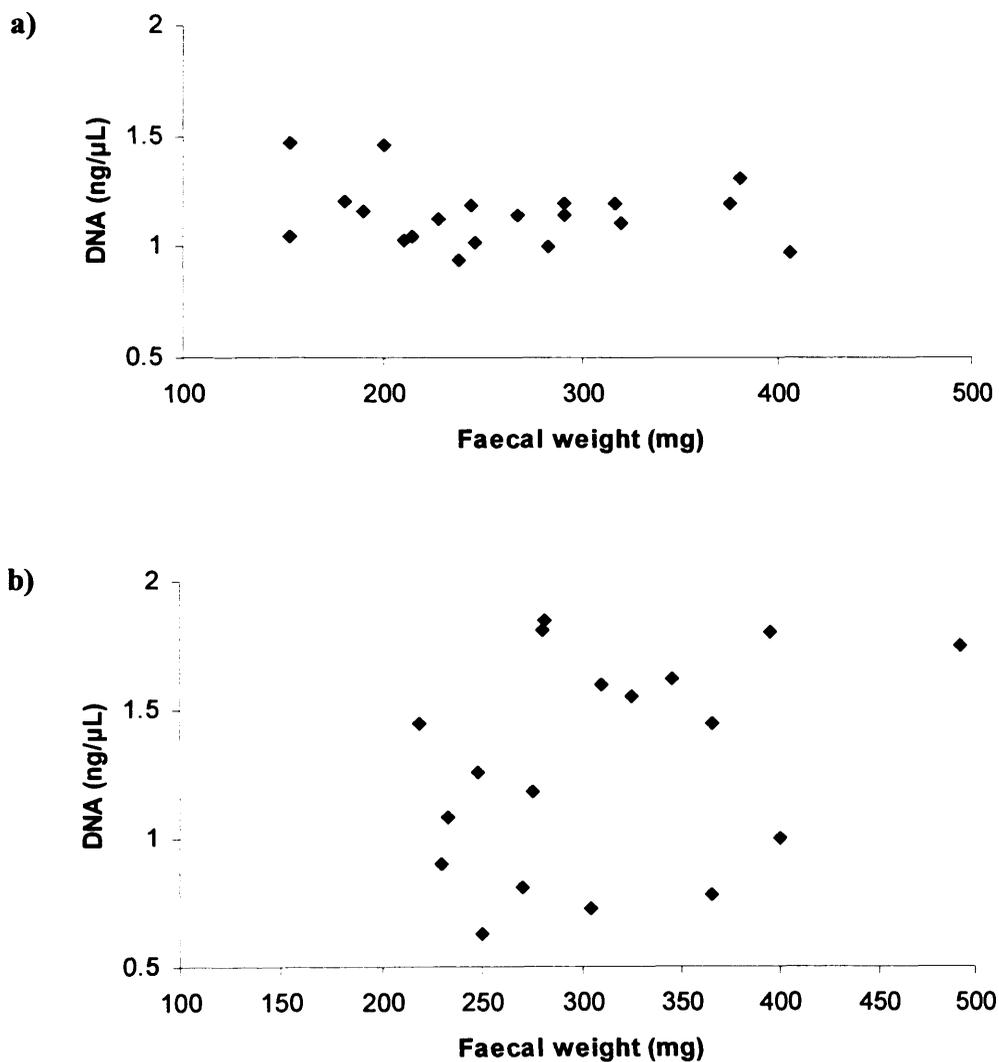
Species	Order, Family	C1-J-2195 / C1-N-2329 = 134 bp		C1-J-3400 / C1-N-3494 = 94 bp		C1-J-3400 / C1-N-3661 = 261 bp		C1-J-3571 / C1-N-3666 = 90 bp	
		50 °C	55 °C	50 °C	55 °C	50 °C	55 °C	50 °C	55 °C
Mite	Acaridae sp. (Acarina: Acaridae)	-	-	√	√	√	√	-	-
Spider	<i>Erigone dentipalpis</i> (Araneae: Linyphiidae)	-	-	-	-	√	√	-	-
Ground beetle	<i>Notiophilus biguttatus</i> (Coleoptera: Carabidae)	√	√	√	-	√	√	√	√
Leaf beetle	<i>Oulema melangus</i> (Coleoptera: Chrysomelidae)	-	√	-	-	-	-	-	-
Lady beetle	<i>Adalia bipunctata</i> (Coleoptera: Coccinellidae)	-	-	-	√	√	√	-	-
Rove beetle	<i>Tachyporus obtusus</i> (Coleoptera: Staphylinidae)	-	-	-	-	-	-	-	-
Earwig	<i>Forficula</i> sp. (Dermaptera: Forficulidae)	-	-	-	-	-	-	-	-
Hoverfly	<i>Scaeva pyrausti</i> (Diptera: Syrphidae)	-	-	-	-	-	-	-	-
Hoverfly	<i>Metasyrphus luniger</i> (Diptera: Syrphidae)	-	-	-	√	-	-	-	-
Cranefly	Tipulidae sp. (Diptera: Tipulidae)	-	-	-	-	√	√	-	-
Earthworm	<i>Lumbricus terrestris</i> (Haplotaxida: Lumbricidae)	√	-	-	-	√	√	-	-
Earthworm	<i>Aporrectodea caliginosa</i> (Haplotaxida: Lumbricidae)	√	-	-	-	√	√	-	-
Earthworm	<i>Aporrectodea longa</i> (Haplotaxida: Lumbricidae)	-	-	-	-	√	√	-	-
Earthworm	<i>Lumbricus rubellus</i> (Haplotaxida: Lumbricidae)	-	-	-	-	√	√	-	-
Earthworm	<i>Aporrectodea rosae</i> (Haplotaxida: Lumbricidae)	-	-	-	-	√	√	-	-
Earthworm	<i>Aporrectodea chlorotica</i> (Haplotaxida: Lumbricidae)	-	-	-	-	√	√	-	-
Aphid	<i>Aphis fabae</i> (Hemiptera: Aphididae)	√	√	-	-	√	-	-	-
Aphid	<i>Rhopalosiphum padi</i> (Hemiptera: Aphididae)	-	-	-	-	-	-	-	-
Spittlebug	Formicidae sp. (Hymenoptera: Formicidae)	-	-	√	-	-	√	-	-
Moth	<i>Galleria mellonella</i> (Lepidoptera: Pyralidae)	-	-	-	-	-	-	-	-
Slug	<i>Deroceras reticulatum</i> (Pulmonata: Agriolimacidae)					√	√		
Slug	<i>Arion intermedius</i> (Pulmonata: Arionidae)					√	-		
Slug	<i>Arion owenii</i> (Pulmonata: Arionidae)					√	√		
Slug	<i>Arion distinctus</i> (Pulmonata: Arionidae)	-	-	-	-	√	-	√	-
Slug	<i>Arion hortensis</i> (Pulmonata: Arionidae)	√	-	-	√	√	√	-	-
Slug	<i>Limax flavus</i> (Pulmonata: Limacidae)	-	-	-	-	-	-	-	-
Thrip	Thripidae sp. (Thysanoptera: Thripidae)	√	√	-	√	-	-	-	-
Chicken	<i>Gallus gallus</i> (Galliformes: Phasianidae)	√	√	-	-	-	-	-	-
Mouse	<i>Mus musculus</i> (Rodentia: Muridae)	-	-	-	-	√	-	-	-
Slow worm	<i>Anguis fragilis</i> (Sauria: Anguidae)	√	√	-	√	√	√	-	-
Smooth snake	<i>Coronella austriaca</i> (Squamata: Colubridae)	√	√	-	√	-	-	√	√
Garter snake	<i>Thamnophis marcianus</i> (Squamata: Natricidae)	-	-	-	√	√	-	-	-
		<b>31%</b>	<b>24%</b>	<b>10%</b>	<b>28%</b>	<b>63%</b>	<b>50%</b>	<b>10%</b>	<b>6%</b>

Species	Order, Family	C1-J-1891 / C1-N-2191 = 300 bp	C1-J-1891 / C1-N-2093 = 202 bp	C1-J-1891 / C1-N-2105 = 214 bp	C1-J-1894 / C1-N-2191 = 297 bp	C1-J-1894 / C1-N-2093 = 199 bp	C1-J-1894 / C1-N-2105 = 211 bp
		50 °C					
Mite	Acaridae sp. (Acarina: Acaridae)						
Spider	<i>Erigone dentipalpis</i> (Araneae: Linyphiidae)	-	-	-	-	-	-
Ground beetle	<i>Notiophilus biguttatus</i> (Coleoptera: Carabidae)	√	-	-	-	-	-
Leaf beetle	<i>Oulema melangus</i> (Coleoptera: Chrysomelidae)						
Lady beetle	<i>Adalia bipunctata</i> (Coleoptera: Coccinellidae)	-	√	√	-	-	-
Rove beetle	<i>Tachyporus obtusus</i> (Coleoptera: Staphylinidae)	-	-	-	-	√	-
Earwig	<i>Forficula</i> sp. (Dermaptera: Forficulidae)	-	-	-	-	-	-
Hoverfly	<i>Scaeva pyrausti</i> (Diptera: Syrphidae)						
Hoverfly	<i>Metasyrphus luniger</i> (Diptera: Syrphidae)						
Cranefly	Tipulidae sp. (Diptera: Tipulidae)	-	-	-	√	√	-
Earthworm	<i>Lumbricus terrestris</i> (Haplotaxida: Lumbricidae)	-	-	-	√	-	-
Earthworm	<i>Aporrectodea caliginosa</i> (Haplotaxida: Lumbricidae)	-	-	-	-	√	√
Earthworm	<i>Aporrectodea longa</i> (Haplotaxida: Lumbricidae)	-	-	-	-	-	-
Earthworm	<i>Lumbricus rubellus</i> (Haplotaxida: Lumbricidae)	-	-	-	√	-	√
Earthworm	<i>Aporrectodea rosae</i> (Haplotaxida: Lumbricidae)	-	-	-	-	-	-
Earthworm	<i>Aporrectodea chlorotica</i> (Haplotaxida: Lumbricidae)	-	-	-	-	-	-
Aphid	<i>Aphis fabae</i> (Hemiptera: Aphididae)						
Aphid	<i>Rhopalosiphum padi</i> (Hemiptera: Aphididae)						
Spittlebug	Formicidae sp. (Hymenoptera: Formicidae)	-	-	-	-	-	-
Moth	<i>Galleria mellonella</i> (Lepidoptera: Pyralidae)						
Slug	<i>Deroceras reticulatum</i> (Pulmonata: Agriolimacidae)	-	-	-	-	-	-
Slug	<i>Arion intermedius</i> (Pulmonata: Arionidae)	-	-	-	-	√	-
Slug	<i>Arion owenii</i> (Pulmonata: Arionidae)	√	-	-	√	√	-
Slug	<i>Arion distinctus</i> (Pulmonata: Arionidae)	-	-	-	-	√	-
Slug	<i>Arion hortensis</i> (Pulmonata: Arionidae)	-	-	-	√	√	-
Slug	<i>Limax flavus</i> (Pulmonata: Limacidae)	-	-	-	-	-	-
Thrip	Thripidae sp. (Thysanoptera: Thripidae)						
Chicken	<i>Gallus gallus</i> (Galliformes: Phasianidae)						
Mouse	<i>Mus musculus</i> (Rodentia: Muridae)						
Slow worm	<i>Anguis fragilis</i> (Sauria: Anguidae)	-	-	-	-	-	√
Smooth snake	<i>Coronella austriaca</i> (Squamata: Colubridae)	-	-	-	-	-	-
Garter snake	<i>Thamnophis marcianus</i> (Squamata: Natricidae)						
		10%	5%	5%	24%	33%	14%

	Species	Order, Family	C1-N-2191 = 286 bp	C1-N-2105 = 200 bp	C1-N-2191 = 255 bp	HCO2198 = 262 bp	C1-N-2093 = 145 bp	C1-N-2105 = 157 bp
			50 °C	50 °C	50 °C	50 °C	50 °C	50 °C
Mite	Acaridae sp.	(Acarina: Acaridae)						
Spider	<i>Erigone dentipalpis</i>	(Araneae: Linyphiidae)	-	-	-	-	-	√
Ground beetle	<i>Notiophilus biguttatus</i>	(Coleoptera: Carabidae)	√	-	√	-	√	√
Leaf beetle	<i>Oulema melangus</i>	(Coleoptera: Chrysomelidae)						
Lady beetle	<i>Adalia bipunctata</i>	(Coleoptera: Coccinellidae)	-	-	-	-	√	-
Rove beetle	<i>Tachyporus obtusus</i>	(Coleoptera: Staphylinidae)	√	-	-	-	-	-
Earwig	Forficula sp.	(Dermaptera: Forficulidae)	-	-	-	-	-	-
Hoverfly	<i>Scaeva pyrausti</i>	(Diptera: Syrphidae)						
Hoverfly	<i>Metasyrphus luniger</i>	(Diptera: Syrphidae)						
Crane fly	Tipulidae sp.	(Diptera: Tipulidae)	√	-	-	-	√	√
Earthworm	<i>Lumbricus terrestris</i>	(Haplotaxida: Lumbricidae)	-	-	-	-	√	-
Earthworm	<i>Aporrectodea caliginosa</i>	(Haplotaxida: Lumbricidae)	-	√	-	-	-	-
Earthworm	<i>Aporrectodea longa</i>	(Haplotaxida: Lumbricidae)	√	-	-	-	-	-
Earthworm	<i>Lumbricus rubellus</i>	(Haplotaxida: Lumbricidae)	-	-	-	-	-	-
Earthworm	<i>Aporrectodea rosae</i>	(Haplotaxida: Lumbricidae)	-	-	-	-	-	-
Earthworm	<i>Aporrectodea chlorotica</i>	(Haplotaxida: Lumbricidae)	-	-	-	-	-	-
Aphid	<i>Aphis fabae</i>	(Hemiptera: Aphididae)						
Aphid	<i>Rhopalosiphum padi</i>	(Hemiptera: Aphididae)						
Spittlebug	Formicidae sp.	(Hymenoptera: Formicidae)	√	-	√	-	√	√
Moth	<i>Galleria mellonella</i>	(Lepidoptera: Pyralidae)						
Slug	<i>Deroceras reticulatum</i>	(Pulmonata: Agriolimacidae)	√	-	-	-	-	-
Slug	<i>Arion intermedius</i>	(Pulmonata: Arionidae)	√	-	√	-	-	-
Slug	<i>Arion owenii</i>	(Pulmonata: Arionidae)	√	√	√	-	-	√
Slug	<i>Arion distinctus</i>	(Pulmonata: Arionidae)	√	√	√	-	√	√
Slug	<i>Arion hortensis</i>	(Pulmonata: Arionidae)	√	-	√	-	√	-
Slug	<i>Limax flavus</i>	(Pulmonata: Limacidae)	-	-	√	-	√	-
Thrip	Thripidae sp.	(Thysonoptera: Thripidae)						
Chicken	<i>Gallus gallus</i>	(Galliformes: Phasianidae)						
Mouse	<i>Mus musculus</i>	(Rodentia: Muridae)						
Slow worm	<i>Anguis fragilis</i>	(Sauria: Anguidae)	-	-	-	-	-	-
Smooth snake	<i>Coronella austriaca</i>	(Squamata: Colubridae)	-	-	-	-	-	-
Garter snake	<i>Thamnophis marcianus</i>	(Squamata: Natricidae)						
			48%	14%	33%	0%	38%	29%

Table 2.7. COI universal primers tested against a range of taxa (√ indicate amplification success).

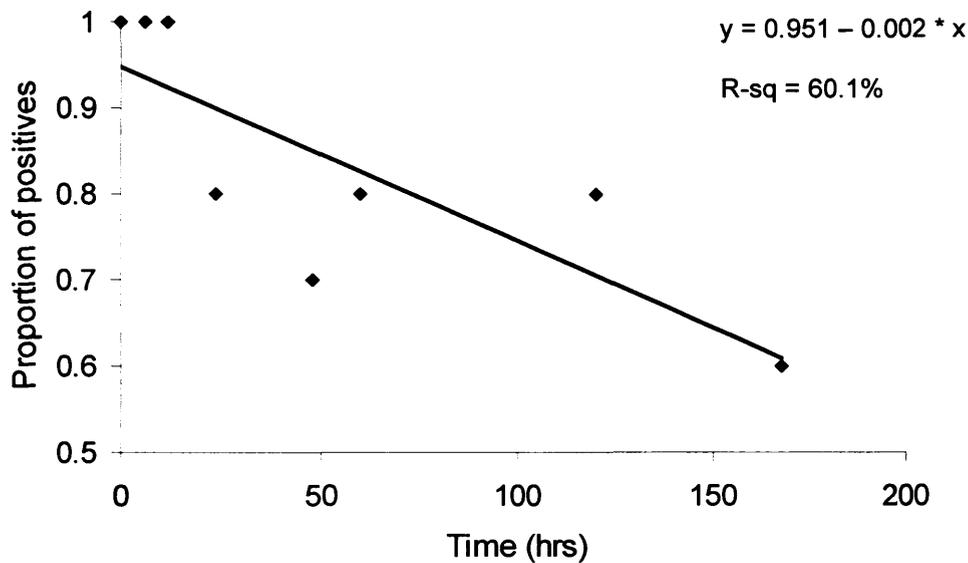
period were omitted. Following DNA extraction, samples were amplified in PCR with *L. terrestris*-specific primers COII-Lt-F2 and COII-Lt-R2 (using the conditions described in Section 2.2). Amplification success was visualized by gel electrophoresis stained with ethidium bromide.



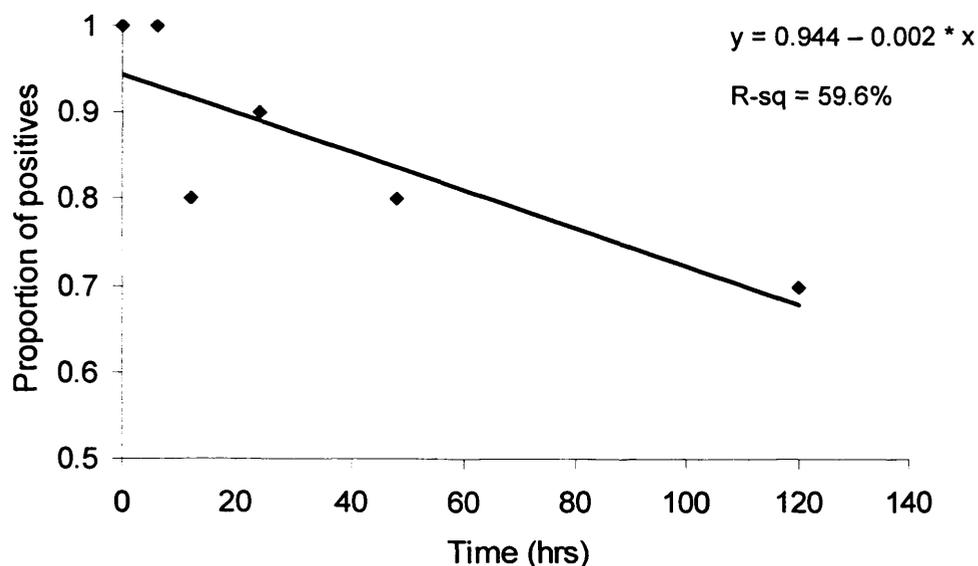
**Figure 2.4.** Relationship between initial faecal weight and DNA concentration following extraction, for a) garter snakes ( $n=20$ ), and b) slow worms ( $n=18$ ).

The detection rate for prey (*L. terrestris*) in the faeces of garter snakes and slow worms significantly decreased with faecal age (Figs 2.5 and 2.6 respectively).

These experiments demonstrate that it is not necessary to standardize faecal weight and that collecting samples within 12 h of defecation is sufficient for a >0.9 probability of prey detection.



**Figure 2.5.** Proportion of garter snake faecal samples testing positive for prey (*Lumbricus terrestris*) when screened in PCR with species-specific primers COII-Lt-F2 and COII-Lt-R2 (King *et al.* 2010), showing a significant decrease in detection rate with time since defecation ( $F=11.6$ ,  $DF=1$ ,  $p=0.02$ ).



**Figure 2.6.** Proportion of slow worm faecal samples testing positive for prey (*Lumbricus terrestris*) when screened in PCR with species-specific primers COII-Lt-F2 and COII-Lt-R2 (King *et al.* 2010), showing a significant decrease in detection rate with time since defecation ( $F=8.4$ ,  $DF=1$ ,  $p=0.04$ ).

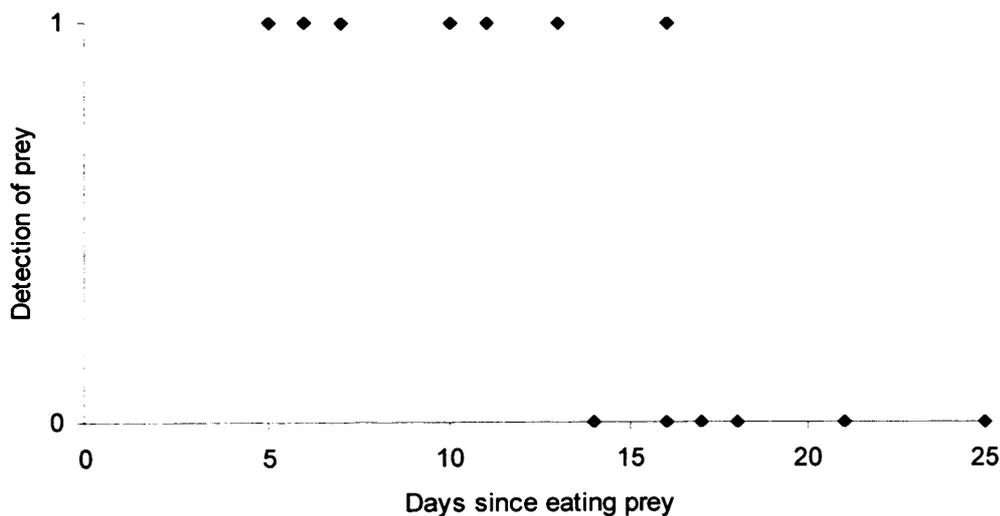
## 2.6 Detection of a pulse of prey in faeces

Garter snakes were fed one meal of mouse (*Mus musculus*) and slow worms fed on slug (*Arion distinctus*), before returning to their standard diet of earthworm (*L. terrestris*). Prey was standardized according to the reptiles' own body weight, with all animals being fed proportionally the same weight food. Enclosures were checked at least every 12 h for three weeks and faecal samples collected in Eppendorf 1.5 mm tubes. Prey DNA was extracted from each faecal sample using the QIAamp® DNA Stool Mini Kit and screened for prey using either mouse (*M. musculus*) specific primers Mus-J-14701 and Mus-N-14905 for snake faeces or *Arion* general primers Ai1F and A2R2 (Harper *et al.* 2005) for slow worms.

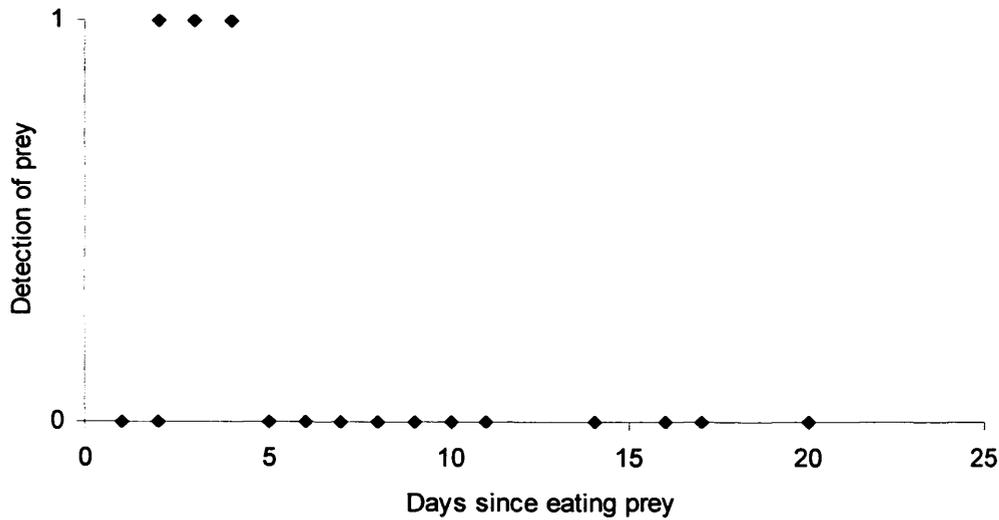
Mouse DNA was detected in garter snake faeces up to sixteen days post-feeding (Fig. 2.7). It was detectable by at least the fifth day, and may have been detectable earlier but no

faecal samples representative of the first four days post-feeding were available (either samples were too poor quality for DNA extraction or defecation had taken place within their water bowl). Slug DNA was detected in slow worm faeces for a much shorter duration, between two and four days post-feeding (Fig. 2.8).

These results suggest that prey may remain detectable for a much longer period in garter snakes than in slow worms, although it may be a function of differences in the efficiency of different primers used. However, primers amplifying similar sized amplicons (approx. 200 BP) were used to try and minimize bias. These results may, therefore, represent the longer, more thorough, digestion by snakes than by lizards. When screening faecal samples collected in the wild this needs to be taken into account. Detecting a diverse range of prey in snakes is therefore more likely than in slow worms, even if their dietary diversity is the same.



**Figure 2.7.** Detection of house mouse (*Mus musculus*) DNA in the faeces of garter snakes up to 25 days post-feeding, using Cytochrome *b* PCR primers Mus-J-14701 and Mus-N-14905.



**Figure 2.8.** Detection of slug (*Arion hortensis*) DNA in the faeces of slow worms up to 20 days post-feeding, using 12S PCR primers Ai1F and A2R2 (Harper *et al.* 2005).

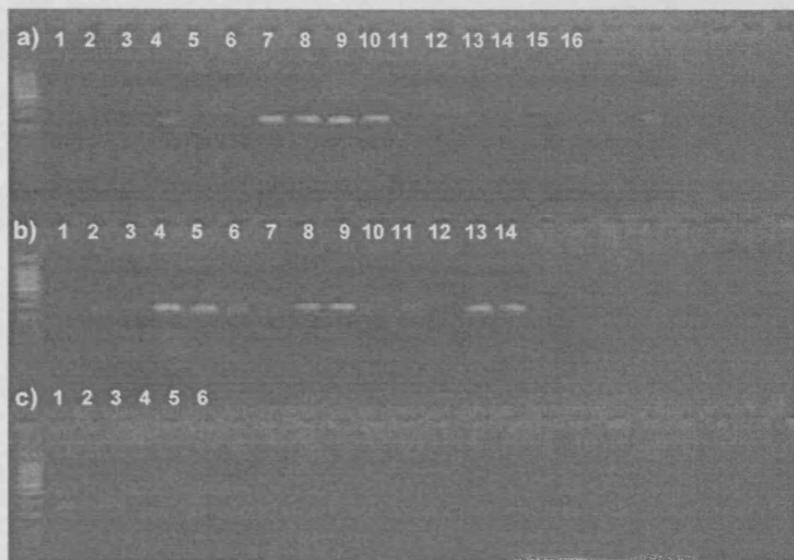
### 2.7 Preliminary cloning of slow worm prey

Fourteen slow worm faecal samples were collected from Ringwood in October 2006. DNA was extracted from each sample using the QIAamp® DNA Stool Mini Kit (Qiagen) in accordance with the manufacturer's instructions. Extracts were amplified in PCR with earthworm-specific 12S primers 185F and 14233R (Harper *et al.* 2005), with 1X buffer, 3 mM MgCl<sub>2</sub>, 0.5 mM dNTP (Invitrogen), 0.1 µM of each primer and 0.48 U *Taq* polymerase (Invitrogen). PCR conditions were 94 °C for 3 min, and 35 cycles of 94 °C for 30 s, 65 °C for 45 s, 72 °C for 45 s, and a final extension at 72 °C for 10 min. Amplification success was visualised by gel electrophoresis stained with ethidium bromide. Eight of the 14 samples produced bands (six strong, two weak, Fig. 2.9). The PCR products for faecal samples four and five (which had produced strong bands) were cloned into the pCR 2.1 TOPO TA cloning vector (Version V, Invitrogen) and transformed into TOP10 chemically competent *Escherichia coli* (Invitrogen). Positive transformants were selected by blue/white screening,

with 40 colonies from each sample picked and suspended in 60  $\mu\text{L}$  of LB medium (containing 0.05 mg/mL ampicillin) and incubated at 37  $^{\circ}\text{C}$  for 1 h. For sequencing, 1  $\mu\text{L}$  of the LB containing each clone was used in PCR with M13 forward and reverse primers (which flank the vector insert site) using 1X buffer, 1.5 mM  $\text{MgCl}_2$ , 2.5 mM dNTP (Invitrogen), 0.375 U *Taq* polymerase (Invitrogen) and 0.5  $\mu\text{M}$  of each primer, with the following conditions: 95 $^{\circ}\text{C}$  for 2 min, then 35 cycles of 95 $^{\circ}\text{C}$  for 1 min, 50 $^{\circ}\text{C}$  for 45 s and 72 $^{\circ}\text{C}$  for 1 min, and a final extension of 72 $^{\circ}\text{C}$  for 10 min. Amplified products were cleaned using ExoSAP in the following reaction: 10  $\mu\text{L}$  of each PCR product, 0.25  $\mu\text{L}$  Exonuclease I, 0.5  $\mu\text{L}$  SAP (shrimp alkaline phosphatase) and incubated for 45 min at 37 $^{\circ}\text{C}$  and 15 min at 80 $^{\circ}\text{C}$ . Cleaned product was then used in sequencing PCR using a Big Dye<sup>TM</sup> terminator sequencing kit (Promega, Madison, WI, USA), using the M13 forward and reverse primers, with products resolved on an ABI 3100 automated capillary DNA analyzer (ABI Prism model 3100, Beaconsfield, UK). Sequence alignments and editing were performed using Sequencer<sup>TM</sup> 4.0 and sequences compared with sequences in the Genbank database using the BLAST algorithm (Altschul *et al.* 1990). Fourteen of the 40 clones from sample four, and 8 from sample five, belonged to earthworm, with the remainder containing primer-dimer. Sequences were compared with closely matching orthologous sequences (>90% shared identity) downloaded from GenBank, and sequences generated directly from a range of potential earthworms, by similarity analysis using neighbour joining algorithm (Saitou & Nei, 1987).

All fourteen sequences for sample four were a 100% match for *Lumbricus rubellus*, an epigeic earthworm species. All eight sequences for sample five were identified as *Aporrectodea caliginosa*, an endogeic earthworm species, with a 99% match.

These findings reveal that a cloning and sequencing approach is an effective method for identifying unknown prey in slow worm diet. The results also highlight that earthworms may be an important component of slow worm diet (with 57% of slow worms having consumed them), and that both surface-living epigeic species and deeper-living endogeic species are present in their diet.



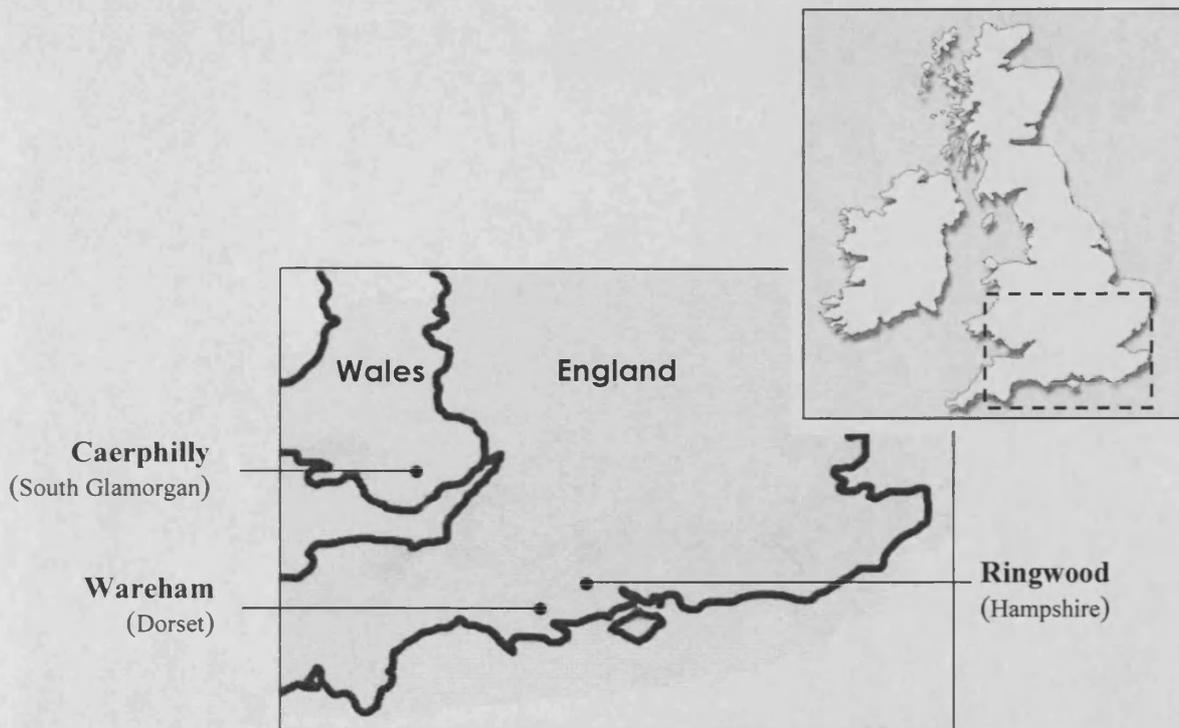
**Figure 2.9.** Agarose gel electrophoresis picture of DNA amplified from slow worm (*Anguis fragilis*) faecal samples with earthworm-specific 12S primers: 185F and 14233R (Harper *et al.* 2005). a) Lanes: 1 (*Aphis fabae*); 2 (*Adalia bipunctata*); 3 (*Erigone dentipalpis*); 4 (*Scaeva pyrausti*); 5 (*Forficula* sp.); 6 (*Tipulidae* sp.); 7 (*Lumbricus terrestris*); 8 (*Aporrectodea caliginosa*); 9 (*Aporrectodea longa*); 10 (*Lumbricus rubellus*); 11 (*Arion intermedius*); 12 (*Deroceras reticulatum*); 13 (*Arion owenii*); 14 (*Limax flavus*); 15 (*Arion hortensis*); 16 (*Metasyrphus luniger*); b) Lanes 1-14: slow worm faecal samples (14 different individuals); c) Lanes 1 and 2: slow worm; lanes 3-6: water (negative control).

## 2.8 Fieldwork

### 2.8.1 Study sites

Three main field sites were used in the study, in Caerphilly, Ringwood and Wareham (Fig. 2.10). The Caerphilly site (51°22'N, 3°13'W) in South Glamorgan, Wales, is an approximately five hectare area of marshy grassland, comprising purple moor grass (*Molinia caerulea*), tufted hair grass (*Deschampsia cespitosa*), gorse (*Ulex* spp.), bramble (*Rubus fruticosus*), various fern species (*Dryopteris* spp.), scattered birch trees (*Betula* spp.) and numerous associated pond flora, surrounded by areas of species-poor acid grassland. The Ringwood site (50°52'N, 1°51'W) in Hampshire, England, is a small area of unimproved grassland adjacent to an approximately four hectare Ericaceous heath consisting of common heather (*Calluna vulgaris*), bell heather (*Erica cinerea*) and gorse (*Ulex* spp.) surrounded by coniferous woodland. The Wareham (50°39'N, 2°06'W) site in Dorset, England, is in a large area of Ericaceous heath (>10 acres), comprising common heather (*C. vulgaris*), bell heather (*E. cinerea*) and gorse (*Ulex* spp.), and marshy grassland bordering a stream, including species such as tufted hair grass (*D. cespitosa*).

These three main sites were visited each month between April and September, 2007 and 2008. Ad hoc visits were made to other sites, including Flat Holm and East Cowes. Flat Holm (51°22'N, 3°07'W), in the Bristol Channel, is a 24 hectare island dominated by coarse grass species such as false oat grass (*Arrhenatherum elatius*), with patches of species-rich red fescue (*Festuca rubra*) dominated maritime grassland occurring close to the cliffs and an absence of any mature trees. The East Cowes site is an abandoned riverside pasture, dominated by grasses and ferns (*Dryopteris* spp.).



**Figure 2.10.** The three main field sites of the study, visited monthly between April and October, 2007 and 2008.

### 2.8.2 Sampling regime

Smooth snakes (*Coronella austriaca*), adders (*Vipera berus*), grass snakes (*Natrix natrix*) and slow worms (*Anguis fragilis*) were located at field sites by monitoring suitable basking areas (south-facing slopes, banks, gullies) and by checking under lightweight corrugated iron sheeting (tins) or asphalt felt, exposed to the sun, which were placed out to attract them.

Animals were caught by hand and placed in a flat plastic box (secured with foam to hold them in place) where they were measured (snout-vent and tail lengths) and were then weighed using a spring balance. Animals were sexed, and head patterning photographed to

enable individual identification. Faecal material was collected where possible. Handling frequently caused them to defecate; failing this, gentle palpation of the animal's abdomen often stimulated defecation (e.g., Goddard, 1984; Monney, 1990; Luiselli & Agrimi, 1991; Rugiero *et al.*, 1995; Drobenkov, 1995). Faeces was collected directly into 1.5ml Eppendorf tubes and stored with 70% ethanol. Any faecal material which fell onto the ground was discarded to avoid contamination.

To avoid pseudo replication in statistical analyses, attempts were made to identify individual smooth snakes and slow worms. Morphometric measurements were compared of same-sexed individuals collected from the same sites, and photographs of head patterns (Fig. 2.11) compared, to clarify whether they were likely to be different. Any that were suspected to have already been sampled were not included.



**Figure 2.11.** Variation in head patterns of smooth snakes (*Coronella austriaca*, top) and slow worms (*Anguis fragilis*, bottom).

a) **Ringwood**

	MALE	FEMALE	TOTAL
APRIL	-	-	-
MAY	8	3	11
JUNE	1	1	2
JULY	3	2	5
AUGUST	1	2	3
SEPTEMBER	4	1	5

**Wareham**

	MALE	FEMALE	TOTAL
APRIL	-	-	-
MAY	7	1	8
JUNE	-	1	1
JULY	6	2	8
AUGUST	1	1	2
SEPTEMBER	1	1	2

b) **Ringwood**

	MALE	FEMALE	SUBADULT	JUVENILE	TOTAL
APRIL	13	16	17	-	46
MAY	13	13	8	1	35
JUNE	6	23	6	-	35
JULY	18	24	3	-	45
AUGUST	12	16	7	1	36
SEPTEMBER	5	5	-	1	11

**Wareham**

	MALE	FEMALE	SUBADULT	JUVENILE	TOTAL
APRIL	3	5	3	-	11
MAY	4	1	3	2	10
JUNE	3	1	4	-	8
JULY	5	14	5	-	24
AUGUST	3	15	3	1	22
SEPTEMBER	5	5	2	-	12

**Caerphilly**

	MALE	FEMALE	SUBADULT	JUVENILE	TOTAL
APRIL	9	1	1	-	11
MAY	5	-	9	5	19
JUNE	2	4	10	2	18
JULY	3	12	7	-	22
AUGUST	6	8	4	-	18
SEPTEMBER	8	7	1	-	16

**Table 2.8.** Number of a) smooth snake and b) slow worm faecal samples collected between April and September, 2007 and 2008, from which DNA was successfully extracted.

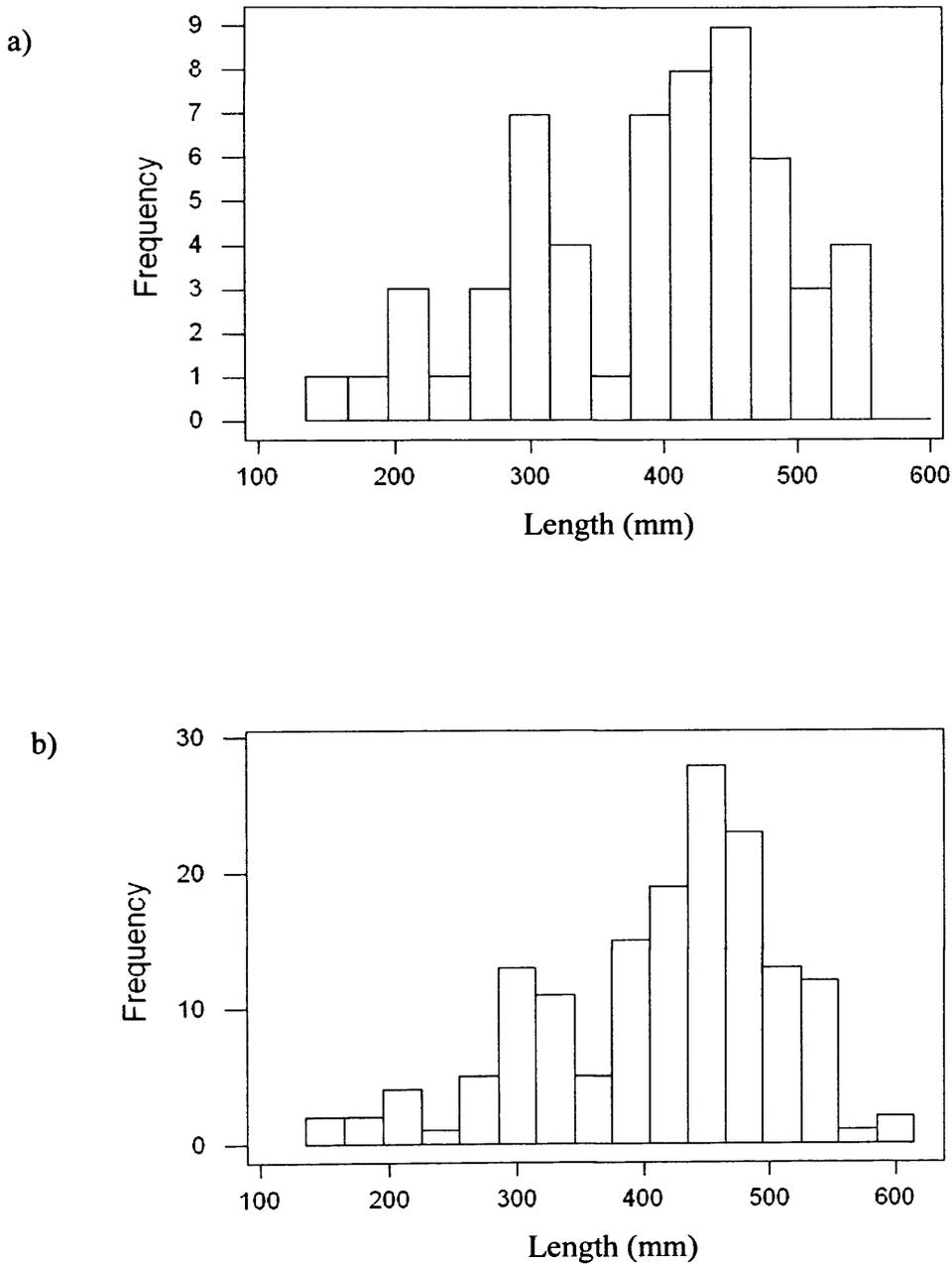
DNA from reptile faeces was extracted using the QIAamp® DNA Stool Mini Kit (Qiagen) in accordance with the manufacturer's instructions. To test for successful extraction of DNA, extracts were amplified in PCR with universal primers LCO1498 (Folmer *et al.* 1994) and C1-N-1770 (using the conditions described in Section 2.3.3.2). In total, 399 faecal samples were collected and extracted from slow worms, 47 from smooth snakes, 14 from grass snakes, and five from juvenile adders. Details of slow worm and smooth snake sampling numbers are shown in Table 2.8.

### 2.8.3 *Descriptive statistics of smooth snakes and slow worms*

The sex ratio of smooth snakes found at Ringwood did not deviate from a 50:50 ratio, whereas at Wareham a significantly higher proportion of male snakes were found (G test:  $n=83$ ,  $G=4.87$ ,  $p=0.03$ ). Sex ratios of slow worms deviated from 50:50 at Ringwood ( $n=182$ ,  $G=3.45$ ,  $p=0.06$ ) and Wareham ( $n=82$ ,  $G=6.54$ ,  $p=0.01$ ) with both showing a higher proportion of females encountered, whereas at Caerphilly the sex ratio was 50:50.

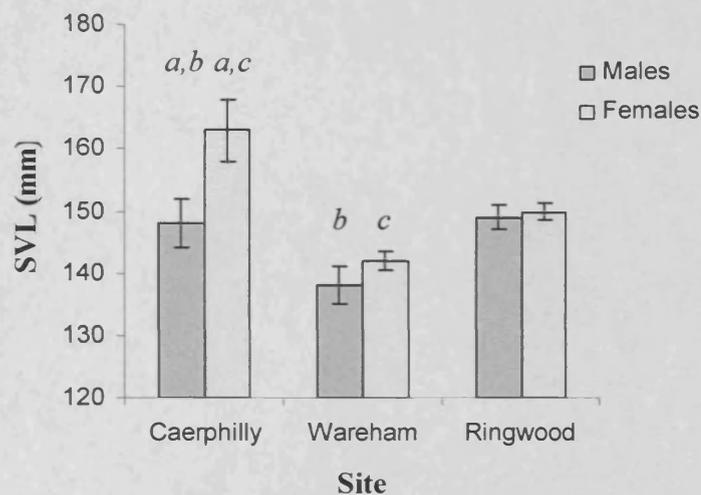
There was no difference in snout-vent length (SVL) between male and female smooth snakes (Mann-Whitney:  $W=4550$ ,  $DF=123$ ,  $p=0.946$ ). The smooth snakes induced to defecate by the palpation method were smaller than those that failed to defecate (Mann-Whitney:  $W=3293$ ,  $DF=138$ ,  $p=0.002$ ), with a median SVL of 340mm for those that defecated and 360mm for those that did not. This is likely a result of larger snakes being stronger and less fearful, and means that dietary analyses in this study may not be truly representative of the entire population. The largest male encountered during this study was 600mm (total length) and the largest female 550mm, with faecal samples were obtained from males up to 540mm and females up to 550mm (which includes the largest female

found). Frequency distributions of the total length of smooth snakes in the population is shown in Fig. 2.12a, with Fig. 2.12b. Most size classes in the population were represented in the study.



**Figure 2.12.** Frequency distributions for smooth snake length (total length, mm) from a) the whole population encountered; and b) snakes from which faeces was obtained.

There was a significant difference in length (SVL) at the three different field sites for male slow worms (Kruskal-Wallis:  $n=142$ ,  $H=6.58$ ,  $DF=2$ ,  $p=0.04$ ) and female slow worms (Kruskal-Wallis:  $n=184$ ,  $H=18.00$ ,  $DF=2$ ,  $p<0.001$ ) (Fig. 2.13), with males and females in Caerphilly being longer than those in Wareham. Furthermore, female slow worms at Caerphilly were significantly longer than males (Mann Whitney:  $n=68$ ,  $W=1126$ ,  $p=0.02$ , Fig. 2.13).



**Figure 2.13.** Length of male and female slow worms at three different sites, showing significant differences between them ( $a = p<0.05$ ;  $b = p<0.01$ ;  $c = p<0.001$ ).

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## **Chapter 3**

### **Methods for preventing predator bias in PCR**

### 3.1 Abstract

Polymerase chain reaction (PCR) is becoming an increasingly popular tool for dietary analysis. Detection of short fragments of multicopy prey DNA/RNA in guts or faeces of predators allows diet to be established even where morphology is destroyed rendering visual identification misleading or impossible. While many studies have employed species-specific PCR primers to analyse diet for certain prey, this approach relies on *a priori* expectations of what prey may have been eaten. A comprehensive analysis of all prey without any prior expectations can be achieved by amplification with universal PCR primers followed by identification of amplicons through cloning and sequencing or, increasingly, new generation high-throughput sequencing. However, gut contents or faeces commonly contain an excess of predator DNA, sloughed off from the gut or intestinal lining, compared to the digested DNA of prey. Various methods have been developed in an attempt to bias PCR in favour of these rarer prey DNA fragments, including the use of endonuclease restriction digest of predator DNA, and PCR clamping, which involves the synthesis of oligonucleotides which bind to and inhibit amplification of predator DNA.

In this chapter, the use of restriction enzymes and PCR clamping to prevent predator DNA domination in an artificial mixture of dominant (99%) and rare (1%) DNA are explored within the framework of developing a method for detailed analysis of slow worm diet. PCR clamping proved to be a fast and cheap means for the complete removal of specific unwanted DNA templates from a mixture. PCR clamping with just 4 $\mu$ M of modified oligonucleotide was sufficient to bias PCR amplification 100% in favour of the rare DNA. Restriction enzyme digest post-PCR resulted in a very low yield of overall DNA, whereas pre-PCR digest, even using up to 64 units of endonuclease, failed to noticeably reduce predator amplification at all.

### 3.1 Introduction

The use of polymerase chain reaction (PCR) in elucidating the diet of predators (Symondson 2002) has grown in popularity and versatility since it was first applied to identifying bloodmeals consumed by haematophagous insects (Coulson *et al.* 1990; Tobolewski *et al.* 1992; Gokool *et al.* 1993) and since its first use in characterizing diet from DNA present in faeces (Hoss *et al.* 1992). By targeting short fragments of multicopy DNA/RNA (commonly mitochondrial DNA or nuclear ribosomal RNA), such techniques allow the DNA of digested prey to be identified even when the morphology is completely destroyed (e.g. Jarman and Wilson 2004; Jarman, Deagle and Wilson 2004; Parsons *et al.* 2005; Deagle *et al.* 2007). This overcomes the biases and limitations of conventional faecal screening or gut dissections which rely on visual identification of morphological remains. A variety of different approaches to identifying prey DNA by PCR have been used, but they can be divided into two broad categories: those strategies which employ taxa-specific (species/group) PCR primers (e.g. Table 3.1a,b) and those which use universal primers (Table 3.1c).

#### 3.1.1 *Taxa-specific primers*

Species- and group-specific PCR primers have been developed to target a wide range of taxa (Table 3.1a,b). The approach involves the design of oligonucleotide primers to complement regions of DNA unique to the target organism(s), most commonly accomplished by alignment of homologous sequences from non-target (and ideally closely-related) species. An alternative is the design of primers from sequence characterized amplified regions (SCARs) derived from a randomly amplified polymorphic DNA (RAPD) band (Agusti *et al.* 2000, 2002; Zhang *et al.*

**a) SPECIES-SPECIFIC PRIMERS**

<b>PREY SPECIES</b>			<b>PREDATOR</b>	<b>GENE</b>	<b>REFERENCE</b>
<i>Emiliana huxleyi</i>	Algae	>	Copepod	18S	Nejstgaard <i>et al.</i> 2003 †
<i>Megoura viciae</i>	Aphid	>	Beetle	COI	Harper <i>et al.</i> 2005 †
<i>Sitobian avenae</i>	Aphid	>	Beetle	COII	Harper <i>et al.</i> 2005 †
<i>Metpolophium</i>	Aphid	>	Beetle	COII	Harper <i>et al.</i> 2005 †
<i>Myzus persicae</i>	Aphid	>	Beetle	COI	Harper <i>et al.</i> 2005 †
<i>Aphis fabae</i>	Aphid	>	Beetle	COI	Harper <i>et al.</i> 2005 †
<i>Sitobian avenae</i>	Aphid	>	Spider	COII	Sheppard <i>et al.</i> 2005
<i>Sitobian avenae</i>	Aphid	>	Beetle	COII	Sheppard <i>et al.</i> 2005
<i>Schizaphis graminum</i>	Aphid	>	Beetle	COII	Chen <i>et al.</i> 2000
<i>Diluraphis noxia</i>	Aphid	>	Beetle	COII	Chen <i>et al.</i> 2000
<i>Rhopalosiphum paid</i>	Aphid	>	Beetle	COII	Chen <i>et al.</i> 2000
<i>Rhopalosiphum maidis</i>	Aphid	>	Beetle	COII	Chen <i>et al.</i> 2000
<i>Sitobian avenae</i>	Aphid	>	Beetle	COII	Chen <i>et al.</i> 2000
<i>Sipha flava</i>	Aphid	>	Beetle	COII	Chen <i>et al.</i> 2000
<i>Aphis fabae</i>	Aphid	>	Beetle	COI	Traugott and Symondson 2008
<i>Aphis fabae</i>	Aphid	>	Spider	COI	Traugott and Symondson 2008
<i>Sitobian avenae</i>	Aphid	>	Beetle	COI	von Berg <i>et al.</i> 2008
<i>Aphis glycines</i>	Aphid	>	Thrip	COI	Harwood <i>et al.</i> 2007 †
<i>Melolontha melolontha</i>	Beetle	>	Beetle	COI	Juen and Traugott 2005
<i>Amphimallon solstitiale</i>	Beetle	>	Beetle	COI	Juen and Traugott 2006
<i>Harmonia axyridis</i>	Beetle	>	Thrip	COI	Harwood <i>et al.</i> 2007 †
<i>Isotoma anglicana</i>	Colembola	>	Spider	COI	Agusti <i>et al.</i> 2003b
<i>Lepidocyrtus cyaneus</i>	Colembola	>	Spider	COI	Agusti <i>et al.</i> 2003b
<i>Entomobrya multifasciata</i>	Colembols	>	Spider	COI	Agusti <i>et al.</i> 2003b
<i>Calanus helgolandicus</i>	Copepod	>	Copepod	COI	Vestheim <i>et al.</i> 2005

**Table 3.1. a)** Studies employing species-specific primers to investigate predation.

† Studies on wild animals.

**a) SPECIES-SPECIFIC PRIMERS (cont)**

<b>PREY SPECIES</b>		<b>PREDATOR</b>	<b>GENE</b>	<b>REFERENCE</b>
<i>Leptinotarsa decemlineata</i>	Coleoptera	Coleoptera	COI	Greenstone <i>et al.</i> 2007
<i>Leptinotarsa decemlineata</i>	Coleoptera	Hemiptera	COI	Greenstone <i>et al.</i> 2007
<i>Leptinotarsa decemlineata</i>	Coleoptera	Hemiptera	COI	Grenstone <i>et al.</i> 2010 †
<i>Leptinotarsa decemlineata</i>	Coleoptera	Coleoptera	COI	Grenstone <i>et al.</i> 2010 †
<i>Gecarciudea natalis</i>	Crab	> Shark	SSU	Meekan <i>et al.</i> 2009 †
<i>Allolobophora chlorotica</i>	Earthworm	> Beetle	COI	King <i>et al.</i> 2010 †
<i>Aporrectodea caliginosa</i>	Earthworm	> Beetle	COI	King <i>et al.</i> 2010 †
<i>Aporrectodea longa</i>	Earthworm	> Beetle	COI	King <i>et al.</i> 2010 †
<i>Lumbricus castaneus</i>	Earthworm	> Beetle	COI	King <i>et al.</i> 2010 †
<i>Lumbricus rubellus</i>	Earthworm	> Beetle	COI	King <i>et al.</i> 2010 †
<i>Lumbricus terrestris</i>	Earthworm	> Beetle	COI	King <i>et al.</i> 2010 †
<i>Paralichthys olivaceus</i>	Fish	> Fish	CR	Saitoh <i>et al.</i> 2003 †
<i>Paralichthys olivaceus</i>	Fish	> Invertebrates	CR	Saitoh <i>et al.</i> 2003 †
<i>Kareius bicoloratus</i>	Fish	> Shrimp	D-loop	Asahida <i>et al.</i> 1997 †
<i>Gadus morhua</i>	Fish	> Seal	16S	Marshall <i>et al.</i> 2010 †
<i>Boreogadus saida</i>	Fish	> Seal	16S	Marshall <i>et al.</i> 2010 †
<i>Mallotus villosus</i>	Fish	> Seal	16S	Marshall <i>et al.</i> 2010 †
<i>Cacopsylla pyricola</i>	Homeoptera	> Arthropods	COI	Agusti <i>et al.</i> 2003a
<i>Vallonia pulcinella</i>	Mollusc	> Beetle	12S	Harper <i>et al.</i> 2005 †
<i>Deroceras reticulatum</i>	Mollusc	> Beetle	12S	Harper <i>et al.</i> 2005 †
<i>Candidula intersecta</i>	Mollusc	> Beetle	12S	Harper <i>et al.</i> 2005 †
<i>Deroceras reticulatum</i>	Mollusc	> Beetle	12S	Foltan <i>et al.</i> 2005
<i>Haliotis rubra</i>	Mollusc	> Lobster	COI	Redd <i>et al.</i> 2008
<i>Culex quinquefasciatus</i>	Mosquito	> Beetle	á-esterase	Zaidi <i>et al.</i> 1999

**Table 3.1 (cont). a)** Studies employing species-specific primers to investigate predation.  
 † Studies on wild animals.

**a) SPECIES-SPECIFIC PRIMERS (cont)**

<b>PREY SPECIES</b>			<b>PREDATOR</b>	<b>GENE</b>	<b>REFERENCE</b>
<i>Plutella xylostella</i>	Moth	>	Damsel bug	ITS-1	Ma <i>et al.</i> 2005
<i>Plutella xylostella</i>	Moth	>	Spider	ITS-1	Ma <i>et al.</i> 2005
<i>Ostrinia nubilalis</i>	Moth	>	Beetle	18S/ITS-1/5.8S	Hoogendoorn and Heimpel 2001
<i>Lysiphlebus testaceipes</i>	Parasitoid	>	Beetle	COI	Traugott and Symondson 2008
<i>Lysiphlebus testaceipes</i>	Parasitoid	>	Spider	COI	Traugott and Symondson 2008
<i>Centrosephanus rodgersii</i>	Sea urchin	>	Lobster	18S	Redd <i>et al.</i> 2008
<i>Heliocidaris erythrogramma</i>	Sea urchin	>	Lobster	18S	Redd <i>et al.</i> 2008
<i>Neohydatothrips variabilis</i>	Thrip	>	Thrip	COI	Harwood <i>et al.</i> 2007 †
<i>Sitona sp.</i>	Weevil	>	Beetle	COI	Harper <i>et al.</i> 2005 †

**Table 3.1 (cont). a)** Studies employing species-specific primers to investigate predation.

† Studies on wild animals.

2007), but this has the disadvantage that target regions will not be multiple-copy and hence have reduced detectability. Following amplification of faecal DNA by PCR, prey identity can then be resolved by sequencing (e.g. Farrell *et al.* 2000) but, more often, by the less costly approach of gel electrophoresis or fragment analysis, assuming the size of the target fragment is known (e.g. Chen *et al.* 2000; Nejstgaard *et al.* 2003; Jarman *et al.* 2004; Kasper *et al.* 2004; Admassu *et al.* 2005; Sheppard *et al.* 2005).

A limitation with this method, however, is that the use of species- or group-specific primers requires some *a priori* knowledge or speculation about what prey might be expected, and this may lead to important prey species being overlooked. Also, if a comprehensive analysis of diet is desired it would necessitate a suite of primers, which are time-consuming to design, optimise and multiplex.

### 3.1.2 Universal primers

‘Universal’ or ‘general’ PCR primers are designed to be complementary to a conserved DNA sequence found in as wide a range of species as possible (Hillis and Dixon 1991; Verma and Singh 2003, Table 3.1c). Their potential was first successfully demonstrated by Hoss *et al.* (1992), using universal primers for the chloroplast *rbcL* gene, in determining which plant species were in the diet of European brown bears (*Ursus arctos*). A more comprehensive study by Kasper *et al.* (2004) produced prey inventories for an introduced and a native social wasp to Australia, using the 16S rRNA gene, which allowed for a comparison of their dietary overlap. Following amplification of a homologous sequence with universal primers, PCR product can be profiled in a number of ways. With a PCR-RFLP (restriction fragment length polymorphism) approach, restriction enzymes are used to produce signature profiles for prey species which can

### c) UNIVERSAL PRIMERS

PREDATOR	PREY	GENE	TAXA RESOLVED BY:	REFERENCE
Amphipod	All	COI	Cloning / Post-PCR endonuclease	Blankenship and Yayanos 2005 †
Beetle	All	cyt b	Direct sequencing / Cloning	Pons 2006 †
Beetle	All invertebrates	COI	TGGE	Harper <i>et al.</i> 2006
Bird (capercaillie)	All plants	trnL	Pyrosequencing	Valentini <i>et al.</i> 2009 † Blankenship and Yayanos 2005 †
Bivalve	All	18S	Cloning / Post-PCR endonuclease	†
Brown bear	All plants	rbcL	Direct sequencing	Hoss <i>et al.</i> 1992 †
Brown bear	All plants	trnL	Pyrosequencing	Valentini <i>et al.</i> 2009 †
Dolphin	All	16S	cloning / Pre-PCR endonuclease	Dunshea 2009 †
Giant squid	All	16S	DGGE	Deagle <i>et al.</i> 2005b †
Grasshopper	All plants	trnL	Pyrosequencing	Valentini <i>et al.</i> 2009 †
Ground sloth (ancient)	All plants	rbcL	Direct sequencing	Hofreiter <i>et al.</i> 2000 †
Kangaroo	All plants	trnL	Diagnostic fragment size	Ho <i>et al.</i> 2010 †
Krill	All	18S	DGGE	Martin <i>et al.</i> 2006 †
Lamb	All plants	trnL	Pyrosequencing	Pegard <i>et al.</i> 2009 †
Lobster	All	18S	Cloning / PNA	Chow <i>et al.</i> 2010 †
Marmot	All plants	trnL	Pyrosequencing	Valentini <i>et al.</i> 2009 †
Slug ( <i>Arion ater</i> )	All plants	trnL	Pyrosequencing	Valentini <i>et al.</i> 2009 †
Slug ( <i>Deroceras reticulatum</i> )	All plants	trnL	Pyrosequencing	Valentini <i>et al.</i> 2009 †
Snail ( <i>Helix aspersa</i> )	All plants	trnL	Pyrosequencing	Valentini <i>et al.</i> 2009 †
Vole	All plants	trnL	Pyrosequencing	Soininen <i>et al.</i> 2009 †
Wasp	All	16S	Direct sequencing	Kasper <i>et al.</i> 2004 †
Weevil	All plants	trnL	Direct sequencing	Navarro <i>et al.</i> 2010 †

**Table 3.1. c)** Studies employing universal primers to investigate predation or herbivory.

† Studies on wild animals.

then be compared against profiles obtained from faecal DNA (e.g. Parsons *et al.* 2005). Alternatives include DGGE (denaturing gradient gel electrophoresis) (e.g. Jarman *et al.* 2004; Martin *et al.* 2006) and TGGE (temperature gradient gel electrophoresis) (Harper *et al.* 2006), which separate PCR amplicons based on their melting points along either a denaturant or temperature gradient respectively. The differences in base pair composition between species results in different melting points. Therefore the number of resulting bands separated out in the gel equate to the number of prey (or taxonomic operational units) thereby providing a measure of diversity. Determining actual prey identity using these methods, however, requires either comparison of banding patterns with that obtained from specific prey, which relies on *a priori* predictions of prey, or by excising and sequencing of bands (e.g. Martin *et al.* 2006). More often, prey identity is established by the isolation and sequencing of discrete prey amplicons achieved through cloning. Clones can either be directly sequenced (e.g. Jarman *et al.* 2004; Blankenship 2005; Deagle *et al.* 2005b; Pons 2006) or, to reduce the amount of clones required to be sequenced, they can be screened using restriction RFLP analysis and only clones with unique profiles sequenced (Suzuki *et al.* 2006). As an alternative to cloning, new generation high-throughput sequencing technology provides an unprecedented amount of sequence data at a relatively low cost (Hudson 2008). The GS FLX platform, for example, is capable of producing 400-600 million base pairs per run with 400-500 base pair reads, and the newer GS Junior offers 35 million high-quality bases per run of 400 base pair reads in a bench top sequencer that is about the size as a laser printer. A frequent problem with the use of universal primers, however, is the tendency for the predator's own DNA (sloughed off from the gut or intestinal lining) to outcompete the prey DNA in PCR due to it being higher quality, less degraded DNA. In Deagle *et al.*'s (2005) examination of the diet of Stellar sea lions (*Eumtopius jubatus*) using faeces,

100% of cloned sequences ( $N=70$ ) contained the sequence belonging to the sea lion, prompting the researchers to adopt a group-specific primer approach instead. In an attempt to determine the diet of giant squid (*Architeuthis* sp.), Jarman *et al.* (2004) encountered the same problem, with 98% of clones ( $N=80$ ) belonging to the predator.

### 3.1.2.1 Removal of predator DNA

#### 1. Restriction enzymes

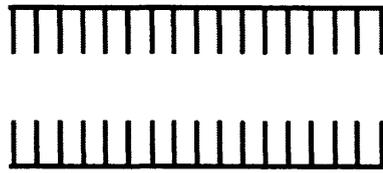
In studying the diet of the bivalve *Lucinuma aequizonata* using universal primers, Blankenship and Yayanos (2005) found that, from three clone libraries, predator DNA accounted for 12.5%, 88% and 100% of the clones. In an attempt to reduce predator swamping in a parallel study on the diet of deep-sea amphipods (*Scopelocheirus schellenbergi* and *Eurythenes gryllus*) PCR products were digested with restriction enzymes for cut sites found in the predator sequence prior to cloning, which resulted in less than 10% of sampled clones containing predator DNA (Blankenship and Yayanos 2005). This was the first published study of an approach designed to subtract predator DNA, and although accomplishing their aim, the method suffers for a couple of reasons. Firstly, by using restriction enzymes after PCR, it failed to prevent predator DNA from outcompeting prey DNA in the reaction, and therefore much of the PCR product was eliminated in the restriction digest and cloning efficiency was very low. Secondly, the restriction enzyme sites were not specific to the predator sequence, but were found to be present in up to 60% of the 150 metazoan taxa they examined. As such the results would have highly biased and underestimated dietary diversity.

A more efficient approach may be the comparison of homologous sequences of the predator with a range of prey taxa to discover unique restriction sites, and the digestion of faecal DNA *prior* to PCR, to reduce amplification bias of predator

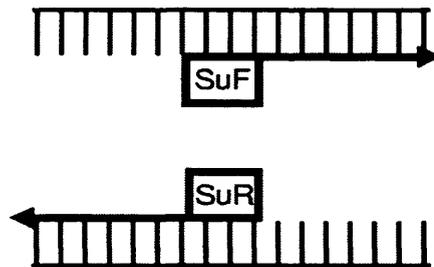
amplicons. Dunshea (2005) applied such a technique in an analysis of bottlenose dolphin (*Tursiops truncatus*) faeces with 16S universal primers using an enzyme with a restriction cut site found in dolphin (and Carnivora, Microchiropteran and other Odontoceti), but not in a majority of other chordates or arthropods, digesting the DNA prior to PCR. Use of the enzyme prior to PCR followed by cloning resulted in predator DNA comprising as low as 21% of the clones, although a control treatment without the enzyme is not described so its effectiveness cannot be determined.

Another method employing restriction enzymes, developed by Green and Minz (2005), involves the use of target-specific PCR primers to amplify DNA and restriction enzymes to cut the resulting double-stranded target amplicon, while leaving single-stranded non-target “prey” DNA intact (Fig. 3.1). Termed “suicide polymerase endonuclease restriction” (SuPER), the approach was successfully demonstrated by the removal of targeted plastid 16S rRNA genes and enhanced amplification of non-plastid 16S genes from DNA extracted from plant roots (Green and Minz 2005). In the same paper, the technique was also successfully used in targeted suppression of *Streptomyces* spp. in artificial mixtures of bacteria DNA, even when the target comprised as much as 80% of the total DNA. The method depends on stringent primer annealing, DNA polymerase elongation, and thermostable endonuclease restriction all operating at the same temperature. At lower temperatures primers may cross-amplify, therefore leading to restriction of non-target “prey” DNA. At higher temperatures, where primer-stringency is greatest, enzymes may be inefficient or inactivated. This inevitably precludes many restriction enzymes, reducing even further the likelihood of finding a suitable target-specific restriction site; however, in situations where endonucleases meeting the above conditions can be found, the technique offers a simple and cheap means of removing predator DNA.

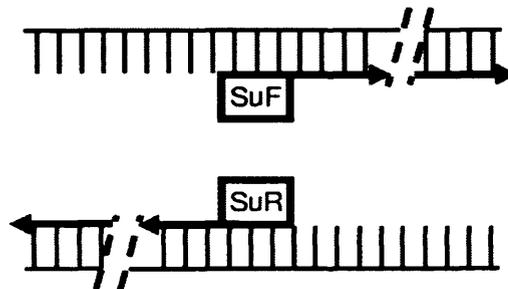
1. Double-stranded DNA is denatured



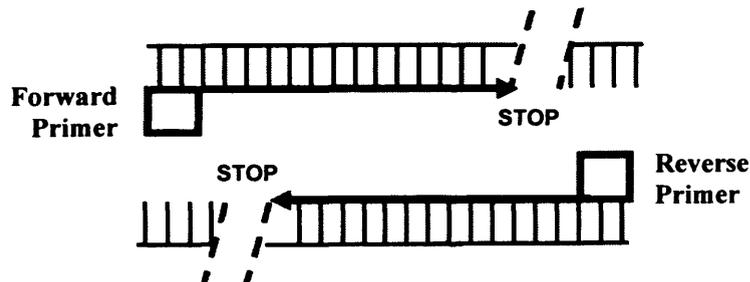
2. SuPER PCR primers (SuF and SuR) anneal to target DNA, and polymerase begins elongation. Primers do not anneal to non-target DNA; therefore, non-target DNA remains single-stranded.



3. Restriction endonuclease digests double-stranded DNAs. A short recognition sequence is advantageous to increase the likelihood of restriction.



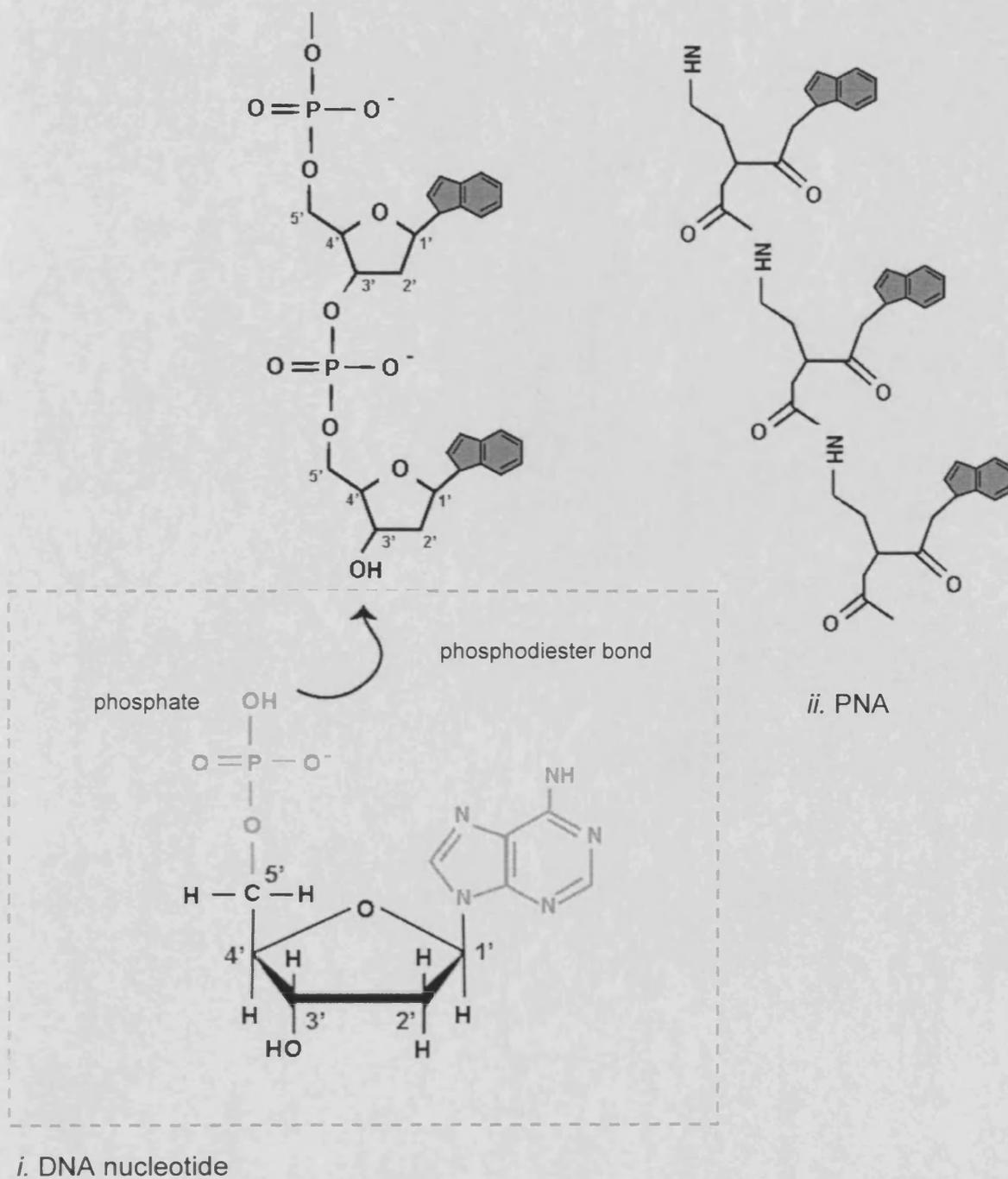
4. DNA digested by SuPER method can no longer be PCR amplified.



**Figure 3.1.** Diagram of the SuPER PCR method (diagram from Green and Minz, 2005).

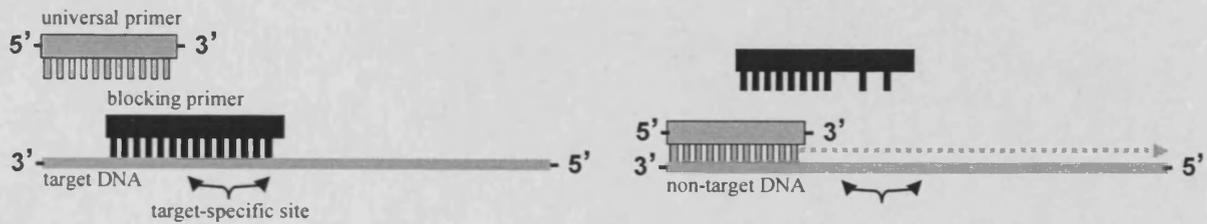
## 2. Selective PCR inhibition

An alternative strategy to destroying predator DNA is to prevent its amplification in PCR. Peptide nucleic acid (PNA), an artificially synthesized DNA analogue, has been shown capable of preventing PCR amplification in a sequence-specific manner, termed 'PCR clamping' (Orum *et al.* 1993). Designed by Nielsen's and Buchardt's groups (Nielsen *et al.* 1991; Egholm *et al.* 1993), PNA comprises of a backbone of repeating N-(2-aminoethyl)-glycine units linked by peptide bonds, with purine and pyrimidine bases joined to the backbone by methylene carbonyl bonds (Nielsen *et al.* 1991; Egholm *et al.* 1993; Corey 1997; Nielsen and Egholm 1999) (Fig. 3.2). As PNA contains no charged phosphate groups, the lack of electrostatic repulsion results in PNAs binding to their complementary nucleic acid sequences with far greater specificity and thermal stability than do their corresponding deoxyribonucleotides (Shakeel *et al.* 2006). Since they are unable to function as primers, they can effectively block PCR amplification of a target sequence differing by as little as a single base pair mismatch (Shakeel *et al.* 2006), and consequently can be used in biasing PCR in favour of non-target "prey" sequences. This can be achieved either by designing PNA sites which overlap with the primer site ('primer exclusion') or which are sited in between primer sites and act by hindering polymerase read-through ('elongation arrest') (Fig. 3.3a,b). While current application is more commonly found in antigene and antisense therapy (Hanvey *et al.*, 1992; Nielsen 1999; Demidov 2002a) or as probes for the detection of genetic disease or viral or bacterial infections (Orum *et al.*, 1993; Lansdorp *et al.* 1996; Demidov 2002b; Igloi 2003; Stender 2003), it has also been used to investigate microbial diversity by allowing suppression of unwanted bacteria or known contaminants (e.g., von Wintzingerode *et al.* 2000; Kimura *et al.* 2006). Utilising the higher stability of PNA/DNA binding Orum *et al.* (1993) incorporated a distinct PNA annealing step into the usual 3 step PCR cycle,

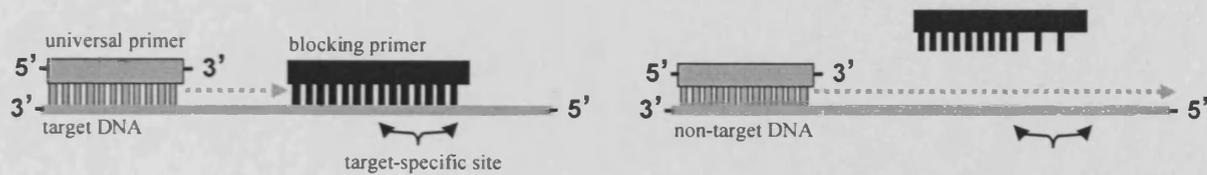


**Figure 3.2.** *i.* The structure of DNA; *ii.* The structure of Peptide nucleic acid (PNA) which can bind to a homologous DNA sequence but cannot be extended by polymerase due to the absence 3' hydroxyl group.

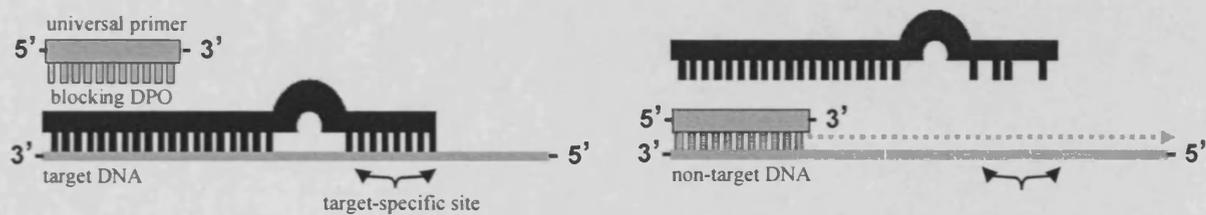
## a) Blocking primer annealing



## b) Blocking primer extension

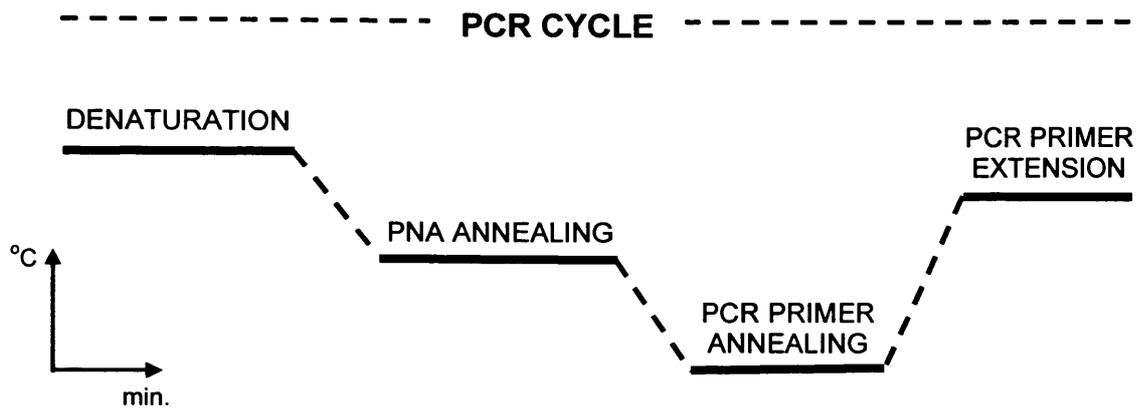


## c) Blocking primer annealing using DPO



**Figure 3.3.** Different approaches using blocking primers to prevent PCR amplification of target DNA templates either by: **a)** Preventing universal primer annealing; **b)** Preventing universal primer extension; or, **c)** Preventing universal primer annealing using a long dual priming oligonucleotide (DPO). Left panel = target DNA; right panel = non-target DNA.

preceding PCR primer annealing and at a temperature which only permitted PNA binding (Fig. 3.4). They found amplification of the target sequence was suppressed in the presence of PNA at just one and a half times the concentration of the universal primers. There is clear potential for its use in dietary studies for biasing PCR reactions in favour of prey amplification, although currently the only study to utilise PNA is by Chow *et al.* (2010) in an examination of Japanese spiny lobster (*Panulirus japonicus*) with universal



**Figure 3.4.** Schematic representation of the PCR cycle profile used in Peptide nucleic acid (PNA) directed clamping (reproduced from Orum *et al.* 1993).

primers, where a ‘PNA clamp’ was successfully employed, allowing detection of a variety of prey.

A drawback to the use of PNA, and other DNA analogues, is the cost of their synthesis. Oligonucleotides with modified 3’ ends, which lack a hydroxyl group and thus prevent polymerase extension, may represent a more cost-effective alternative. Oligonucleotides with phosphate groups added to the 3’ have been shown to work (Liles *et al.* 2003); however, the hydroxyl group may still become partially free and therefore is not as effective (Liles *et al.* 2003). Other candidate modifiers include dideoxynucleotides, aminolinkers or 3’ spacer-C3-CPG (Cradic *et al.* 2004; Dames *et al.* 2007), which are up to a magnitude cheaper to synthesize than PNA, but all work on the basic principle of removing the C3 hydroxyl group.

In an analysis of krill diet, Vestheim and Jarman (2008) demonstrated that complete removal of krill DNA could be achieved by the use of a krill-specific “blocking primer” modified with a 3’ C3 spacer. Using an artificial mixture of krill and algae rDNA in a ratio of 1000:1, amplified in PCR with universal primers and cloned, they found that a 10 times increase in the amount of blocking primer compared to universal primers

reduced the dominant krill template from 100% to as low as 2.2%, and even further when using a higher concentration of blocking primer. The blocking primers were designed to overlap with the universal primer site and prevent primer annealing (i.e. primer exclusion), while another blocking primer designed to anneal to a middle region of the fragment and prevent polymerase elongation (i.e. elongation arrest) resulted in a total absence of PCR product from krill or algae. The authors speculate that by not preventing primer annealing the primers may all have attached to dominant DNA templates and “never found the rarer sequences.” Although it may be more challenging to find predator-specific coding differences directly flanking the universal primer sites, the use of a long dual priming oligonucleotide (DPO) (Chun *et al.* 2007; Vestheim and Jarman 2008) may be used to effectively extend the region flanking the universal primer site for which a blocking primer site can be found (Vestheim and Jarman 2008, Fig. 3.3c). Unlike a long primer (over 25 bases) which can have melting temperatures too high for effective PCR, a DPO, which contains two separate priming regions connected by a polydeoxyinosine linker, is essentially two separate primers with distinct annealing properties, and when modified with a C3 spacer has been found to be as effective as a short blocking primer (Vestheim and Jarman 2008).

In this study we aimed to test two methods for reducing PCR bias in favour of prey DNA: restriction enzyme digest of predator DNA (before or after PCR) and the use of oligonucleotides modified with a 3' C3 spacer (blocking primer) to reduce or prevent PCR amplification of predator DNA. The overall purpose of this chapter was to develop a method for detailed analysis of the diet of slow worms (*Anguis fragilis*) in the wild.

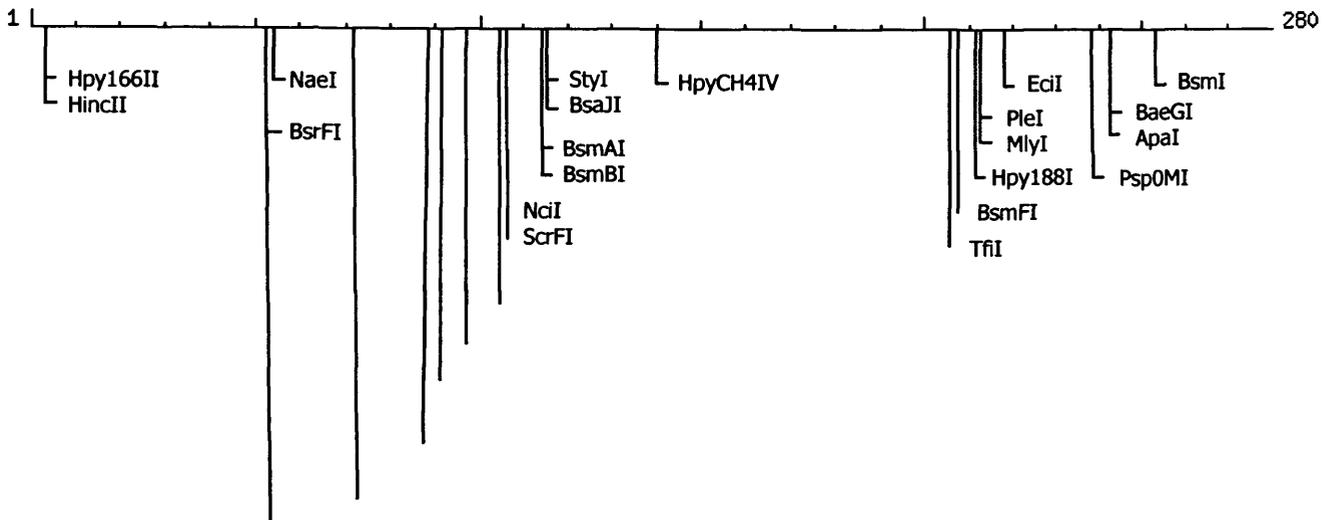
## 3.2 *Method development and Results*

### 3.2.1 *Extraction and sequencing of slow worm DNA*

DNA was extracted from slow worm (*Anguis fragilis*) tissue using the DNeasy® Tissue Kit (Qiagen), following the protocol for animal tissue. A 287 bp fragment of Cytochrome Oxidase I was amplified in PCR with primers LCO1490 (Folmer *et al.* 1994) and C1-N-1777 (described in Section 2.3.3.2) using the following conditions: 1x buffer, 2 mM MgCl<sub>2</sub>, 0.1 mM dNTPs (Invitrogen), 0.5 μM of each primer, 0.45 U *Taq* polymerase (Invitrogen) and 2 μL/25 μL of DNA with an initial denaturation at 94 °C for 3 min, 45 cycles of 94 °C for 30 s, 46 °C for 1 min and 72 °C for 1 min, and a final extension at 72 °C for 5 min. Amplified product was cleaned using ExoSAP in the following reaction: 10 μL of each PCR product, 0.25 μL Exonuclease I, 0.5 μL SAP (shrimp alkaline phosphatase) and incubated for 45 min at 37°C and 15 min at 80°C. Cleaned product was then used in a sequencing PCR using the BigDye™ terminator sequencing kit (Promega, Madison, USA) with sequences resolved on an ABI 3100 automated capillary DNA analyzer (ABI Prism model 3100, Beaconsfield, UK). Forward and reverse sequences were aligned and manually checked for errors using Sequencer™ 4.0. All PCRs were performed on a Peltier Thermal Cycler (Bio-Rad Laboratories, CA, USA).

### 3.2.2 *Restriction enzymes*

All restriction enzyme sites for the COI slow worm fragment were identified using NEBcutter (Vincze, Posfai and Roberts 2003) and are shown in Figure 3.5. Restriction sites for homologous sequences of twenty five different invertebrate taxa (Table 3.2) were established using NEBcutter and any cut sites also found in slow worms were discarded. A range of taxa were used from eleven different orders representative of potential prey, including earthworms and slugs, which slow worms are known to



**Figure 3.5.** Restriction enzyme cut sites for the 280 bp COI fragment of slow worm (*Anguis fragilis*) DNA amplified by LCO1490/C1-J-1777 primers.

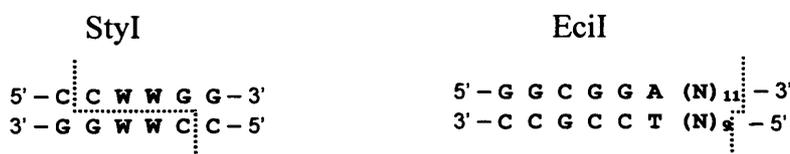
consume (Luiselli 1992). After most restriction sites were excluded for their presence in potential prey taxa, two restriction enzymes specific to slow worm were identified: *StyI* and *EciI*. The cut sites for these are shown in Figure 3.5 and cut sequences in Fig. 3.6.

### 3.2.2.1 Post-PCR restriction enzyme digest

Slow worm DNA was amplified in PCR with universal primers LCO1498 and C1-N-1777 (using conditions described in Section 3.2.1). Amplification success was established by gel electrophoresis stained with ethidium bromide, and DNA concentration quantified using the Nanodrop ND-1000 spectrophotometer. PCR product (100 ng/ $\mu$ L) was then digested by either *EciI* or *StyI* using either 4, 8 or 16 U of enzyme, 1X buffer and 1X BSA, and an incubation period of either 2, 4 or 8 h at 37 °C, followed by 30 min at 65 °C for enzyme inactivation. PCR products were re-visualised by gel electrophoresis to determine whether product had been digested.

Taxa	Species	Accession No.
Haplotaxida	<i>Lumbricus terrestris</i>	DQ092908
Haplotaxida	<i>Aporrectodea caliginosa</i>	DQ092888
Haplotaxida	<i>A. rosea</i>	GUO13835
Haplotaxida	<i>Allolobophora chlorotica</i>	AM774252
Haplotaxida	<i>Dendrodrilus rubidus</i>	DQ092900
Pulmonata	<i>Deroceras reticulatum</i>	FJ917286
Pulmonata	<i>Arion lustanicus</i>	EF535149
Pulmonata	<i>A. rufus</i>	FJ481178
Pulmonata	<i>A. ater</i>	AY987870
Pulmonata	<i>A. subfuscus</i>	GU249587
Pulmonata	<i>Physa pomilia</i>	EU038363
Coleoptera	<i>Pterostichus pennsylvanicus</i>	EU710802
Coleoptera	<i>Poecilus lucublandus</i>	EU710793
Coleoptera	<i>Calosoma scrutator</i>	EU839722
Coleoptera	<i>Henosepilachna vigintioctomaculata</i>	AB002193
Coleoptera	<i>Amara ovata</i>	EU710782
Coleoptera	<i>Staphylinus olens</i>	FJ899826
Isopoda	<i>Ligia hawaiiensis</i>	AY051325
Isopoda	<i>Laevophiloscia yalgooensis</i>	EU364629
Lepidoptera	<i>Scopula ornate</i>	EU443358
Lepidoptera	<i>Bombyx mori</i>	AY048187
Heteroptera	<i>Ilyocoris cimicoides</i>	FJ456947
Heteroptera	<i>Aphelocheirus ellipsoideus</i>	FJ456939
Dermaptera	<i>Labidura riparia</i>	AB435163
Diptera	<i>Drosophila melanogaster</i>	NC_001709
Hemiptera	<i>Orius niger</i>	EU427341
Hymenoptera	<i>Tenthredo mesomela</i>	EF032213
Araneae	<i>Habronattus oregonensis</i>	AY571145

**Table 3.2.** Taxa compared with slow worm in identifying slow worm specific restriction cut sites for COI, using NEBcutter V2.0.



**Figure 3.6.** The cut sites for the two restriction enzymes found to be specific to slow worms (*Anguis fragilis*) for the 287 bp fragment of COI amplified by LCO1490/C1-J-1777 primers.

Treatment with EciI only resulted in partial digestion of PCR product, at all concentrations and incubation periods. Treatment with StyI, however, showed complete digestion using 16 U at incubation periods of 2 h or more. PCR product of the following non-target taxa were also subjected to a 2 h incubation at 37 °C with 16 U of StyI: *Lumbricus terrestris* (Haplotaxida), *L. rubellus* (Haplotaxida), *Aporrectodea caliginosa* (Haplotaxida), *A. longa* (Haplotaxida), *Deroceras reticulatum* (Pulmonata), *Arion intermedius* (Pulmonata), *A. owenii* (Pulmonata), *A. hortensis* (Pulmonata), *A. distinctus* (Pulmonata), *Limax flavus* (Pulmonata), *Tachyporus obtusus* (Araneae), *Erigone dentipalpis* (Araneae), *Forticula* sp. (Dermaptera), *Formicidae* sp. (Hymenoptera), *Notiophilus bigattus* (Coleoptera), *Adalia bipunctata* (Coleoptera), *Aphis fabae* (Hemiptera), *Galleria mellonella* (Lepidoptera), *Tipulidae* sp. (Diptera), and *Scaeva pyrausti* (Diptera). The enzyme failed to cut DNA from any of the non-targets, substantiating its specificity.

To test whether digestion with restriction enzyme StyI could be used to remove unwanted predator (slow worm) DNA and increase detection of prey DNA, a mixture was made comprising 99 ng/μL of slow worm DNA and 1 ng/μL of *Deroceras reticulatum*. DNA was amplified in PCR with LCO1498 and C1-N-1777 primers, checked for amplification success by gel electrophoresis, and digested with 16 U of StyI enzyme for 4 hours at 37 °C. Digested PCR product and non-digested PCR product were each cloned into the pCR 2.1 TOPO TA cloning vector (Version V, Invitrogen), transformed into TOP10 chemically competent *Escherichia coli* (Invitrogen) and positive transformants selected for sequencing by blue/white screening ( $n=96$ ). While cloning from the undigested mixture resulted in an expected 97% slow worm sequences and only 2% *D. reticulatum* sequences, cloning from the digested mixture failed to produce any

colonies. Presumably, as suggested by Blankenship and Yayanos (2005), this was a result of the DNA yield following digestion being too low for efficient transformation.

### 3.2.2.2 *Secondary PCR following enzyme digestion*

In an attempt to overcome the low yield of non-predator DNA following the digestion of predator DNA, a second PCR with LCO1498 and C1-N-1777 primers was performed after the digestion step. However, while complete digestion appeared to have taken place prior to the second PCR, determined by gel electrophoresis, the second PCR re-amplified slow worm DNA and produced an (approximately) equally bright band when again visualised by gel electrophoresis.

### 3.2.2.3 *Pre-PCR enzyme digestion*

Slow worm and *D. reticulatum* DNA were incubated for 4 hours at 37 °C with either 0, 4, 8, 16, 32 or 64 U of StyI, 1X buffer and 1X BSA in the following treatments: 1. Slow worm DNA (20 ng/μL); 2. Slow worm DNA (2 ng/μL); 3. *D. reticulatum* DNA (20 ng/μL); 4. *D. reticulatum* DNA (2 ng/μL), and; 5. A mixture of slow worm DNA (20 ng/μL) and *D. reticulatum* DNA (2 ng/μL). Digested DNA was then amplified in PCR with LCO1498 and C1-N-1777 (using the conditions described in Section 3.2.1) and visualised by gel electrophoresis.

PCR products for all treatments produced strong bands, indicating that StyI had not completely digested all slow worm DNA prior to PCR.

	1490	1540
Slow worm	5'-GGTCAACAAA TCATAAAGAT ATTGGAACCC TCTACCTAAT TTTTGGTGCG-3'	
LCO1490 (forward primer)	GGTCAACAAA TCATAAAGAT ATTGG	
SW-1495	ACAAA TCATAAAGAT ATTGGAACCC TC	
<b>i.</b> <i>Lumbricus terrestris</i>		...T. .A...T.C.. .C....G.T.
<i>Aporrectodea caliginosa</i>		...T. .A..TT.T.. CC.C..C.TC
<b>ii.</b> <i>Physa pomilia</i>		...TT .A..TT.... .....TC
<i>Arion lusitanicus</i>		...TT .A..TT.... .....GATT
<b>iii.</b> <i>Argiope bruennichi</i>		...TT .A...T.G.. .....A..T
<i>Bolyohntes alticeps</i>		TT.TT .A..TT.T.. .....G...
<i>Linyphia triangularis</i>		...TT .G..TT.C.. .....A..T
<i>Habronattus oregonensis</i>		...TT .G..TT.... .....T
<i>Nephila clayata</i>		..AT .A..TT..G. ....G..T
<b>iv.</b> <i>Drosophila melanogaster</i>		...TT .G..TT.... .....T
<i>Tabanus rufofrater</i>		..AT .A..TT.T.. .....G..A
<b>v.</b> <i>Staphylinus olens</i>		...A. .A...T.C.. ...C..A.TA
<i>Monochamus alternatus</i>		...TT .A..TT.T.. .....A
<i>Amara ovata</i>		...TT .A..TT.T.. .....A..A
<i>Pterostichus pennsylvanicus</i>		...TT .A..TT.T.. .....A
<i>Poecilus lucublandus</i>		...TT .A..TT.T.. .....A
<b>vi.</b> <i>Scopula ornata</i>		..AT .A..TT.T.. C..C...ATT
<i>Bombyx mori</i>		..AT .A..TT.T.. .....ATT
<i>Hylephila phyleus</i>		...TT .A..TT.T.. .....ATT
<i>Ostrinia nubilalis</i>		...TT .A..TT.T.. .....AATT
<b>vii.</b> <i>Gerris sp.</i>		...TT .A..TT.T.. A..A...ATA
<i>Ilyocoris cimicoides</i>		...A. .A...T.T.. C....AATC
<i>Orius niger</i>		...TT .A..TT.T.. .....AATA
<i>Aphelocheirus ellipsoideus</i>		..AT .A..TT.TT. A....AATT

**Table 3.3.** Cytochrome Oxidase I alignment of slow worm (*Anguis fragilis*) with homologous sequences of other (potential prey) taxa (haplotaxida (i), pulmonata (ii), araneae (iii), diptera (iv), coleoptera (v), lepidoptera (vi) and hemiptera (vii)) showing matches (.) and mismatches for the region immediately following the forward LCO1490 (Folmer *et al.* 1994) universal primer. Also shown is the slow worm-specific primer SW-1495 which overlaps with the universal primer site.

## 3.2.3 PCR inhibition

## 3.2.3.1 Slow worm primers

The slow worm COI sequence was aligned using BioEdit 7.0.4.1 (Hall, 1999) with homologous sequences obtained from Genbank for a range of different taxa (Table 3.3.) Two primers were designed to be slow worm specific, with the intention of being modified as blocking primers: SW-1495, to be used in conjunction with the C1-N-1777 universal primer to produce a 282 bp fragment, and SW-1730, to be used with universal LCO1490, producing a 240 bp fragment (Fig. 3.7). SW-1495 was designed to overlap with one of the universal primer (LCO1490) sites so that, when modified to not act as a primer, would prevent universal primer annealing. Conversely, SW-1730 did not overlap with universal primer sites and was designed instead to prevent primer extension.



**Figure 3.7.** Slow worm (*Anguis fragilis*) Cytochrome Oxidase I sequence showing universal primers LCO1490 (Folmer *et al.* 1994) and C1-N-1777, and slow worm specific primers SW-1495 and SW-1730.

### 3.2.3.2 *Testing specificity of slow worm primers*

To establish whether slow worm specificity could be achieved at the low annealing temperature and using the long PCR cycle required for universal primers LCO1490 / C1-N-1777, DNA from slow worm and various non-target potential prey taxa were amplified in PCR under the same conditions as described in Section 3.2.1., using DNA from the twenty species listed in Section 3.2.2.1, and primer pairs SW-1495 / C1-N-1777 and LCO1490 / SW-1730. A negative control to test for contamination was included, using water in place of DNA. Amplification success of DNA was confirmed by gel electrophoresis.

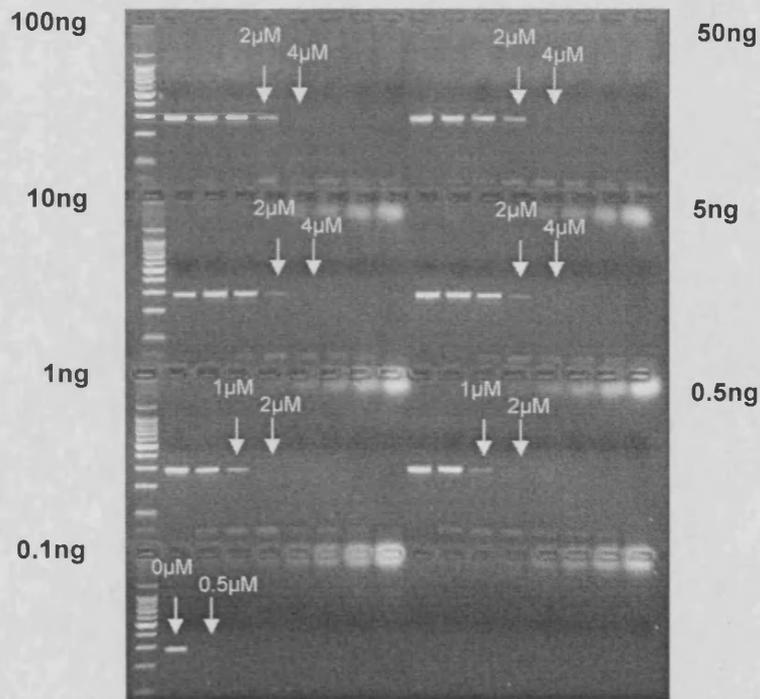
Both sets of primers were specific in amplifying only slow worm DNA and none of the non-target DNAs.

### 3.2.3.3 *Blocking primers*

With target specificity established, the slow worm primers SW-1495 and SW-1730 were synthesized with a 3'-amino modifier C3 CPG, which lacks a hydroxyl group on the 3' necessary for *Taq* polymerase extension. Slow worm DNA of different concentrations (100 ng/ $\mu$ L, 50 ng/ $\mu$ L, 10 ng/ $\mu$ L, 5 ng/ $\mu$ L, 1 ng/ $\mu$ L, 0.5 ng/ $\mu$ L and 0.1 ng/ $\mu$ L, quantified using the Nanodrop ND-1000 spectrophotometer) and a negative control without DNA were amplified in PCR with universal primers LCO1490 and C1-N-1777 (using the conditions described in Section 3.2.1), with and without each of the modified / blocking slow worm primers (SW-1495-C3 and SW-1730-C3) added to the PCR reaction in either 0.5  $\mu$ M, 1  $\mu$ M, 2  $\mu$ M, 4  $\mu$ M, 8  $\mu$ M or 16  $\mu$ M concentrations.

While the blocking primer designed to prevent DNA elongation (SW-1730-C3) failed to prevent slow worm amplification at any concentration of slow worm DNA, even at the highest concentrations of blocking primer, the SW-1495-C3 blocking primer,

designed to prevent the LCO1490 universal primer from annealing, successfully prevented slow worm DNA amplification at all concentrations of slow worm DNA when at least 4  $\mu\text{M}$  of blocking primer was included (Fig. 3.8).



**Figure 3.8.** Amplification of different concentrations of slow worm (*Anguis fragilis*) DNA in PCR with universal primers LCO1490 (Folmer *et al.* 1994) and C1-N-1777 in the presence of blocking primer SW-1495-C3 at concentrations between 0-16  $\mu\text{M}$ .

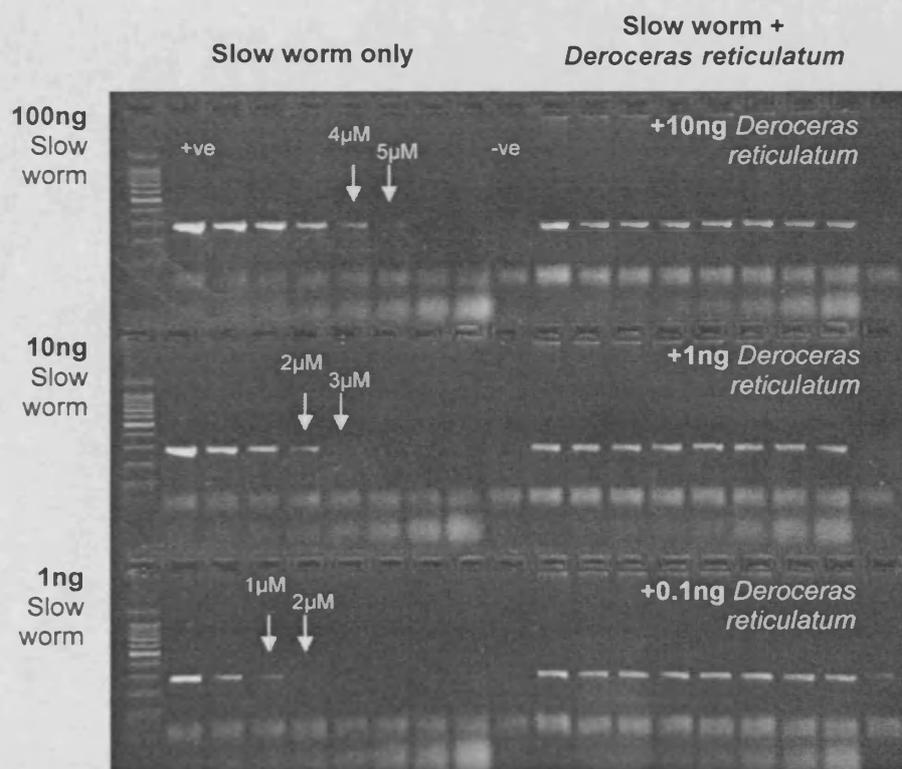
#### 3.2.3.4 Non-target testing of blocking primers

To ensure that the SW-1495-C3 blocking primer would only block slow worm DNA, a PCR was performed to amplify the DNA of the twenty different invertebrate taxa described in Section 3.2.2.1. using the universal LCO1490 / C1-N-1777 primers (conditions described in Section 3.2.1) including 16  $\mu\text{M}$  of SW-1495-C3.

Only slow worm DNA failed to amplify, demonstrating specific blocking of slow worm target DNA.

Slow worm DNA and mixtures of slow worm and non-target “prey” (*D. reticulatum*) DNA were tested, with the non-target “prey” DNA representing 10% of DNA in each mixture (slow worm : *D. reticulatum* DNA, 100:10 ng/ $\mu$ L; 10:1 ng/ $\mu$ L; and 1:0.1 ng/ $\mu$ L). The three different mixtures were amplified in PCR with universal LCO1490 / C1-N-1777 primers and SW-1495-C3 in concentrations of 1, 2, 3, 4, 5, 6 and 7  $\mu$ M, with a positive control (no SW-1495-C3) and a negative control (no DNA).

While slow worm DNA was again successfully blocked at all concentrations of DNA by anything greater than 5  $\mu$ M of SW-1495-C3, the mixtures comprising slow worm DNA and non-target “prey” *D. reticulatum* DNA amplified at all concentrations of SW-1495-C3, suggesting that at higher concentrations of blocking primer only the prey was being amplified (Fig. 3.9).

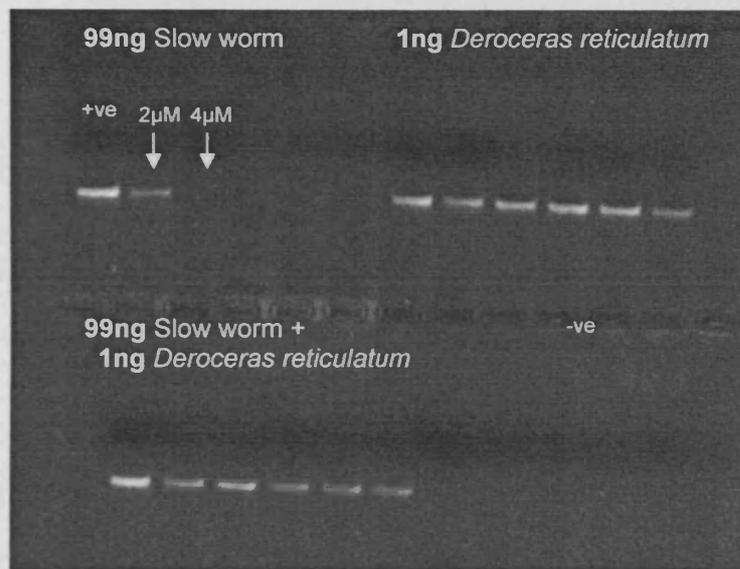


**Figure 3.9.** Amplification of slow worm DNA and mixtures of slow worm (*Anguis fragilis*) DNA and non-target *Deroceras reticulatum* DNA in PCR with universal primers LCO1490 (Folmer *et al.* 1994) and C1-N-1777 in the presence of blocking primer SW-1495-C3 at concentrations between 0-7  $\mu$ M.

### 3.2.3.5 Cloning of artificial mixtures of slow worm and non-target “prey” DNA

A mixture of slow worm and non-target “prey” *D. reticulatum* DNA was made, with a concentration of 99 ng/μL slow worm DNA and 1 ng/μL *D. reticulatum* DNA, and amplified in PCR with universal LCO1490 / C1-N-1777 primers and either 0, 2, 4, 6, 8, 10 μM blocking primer SW-1495-C3 (using conditions described in Section 3.2.1).

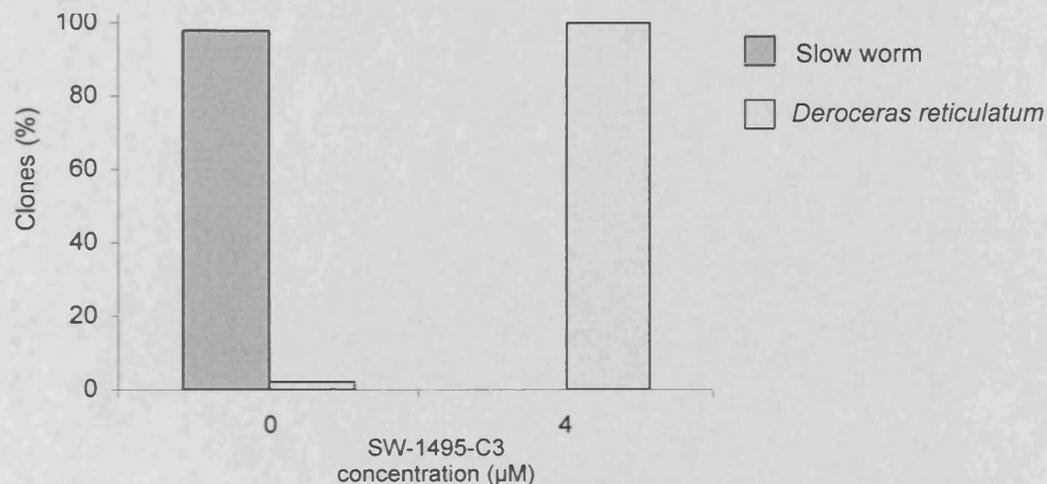
*Deroceras reticulatum* DNA (1 ng/μL) and the mixture of slow worm DNA (100 ng/μL) and *D. reticulatum* DNA (1 ng/μL), amplified *D. reticulatum* DNA at all concentrations of SW-1495-C3 whereas slow worm DNA (99 ng/μL) by itself only amplified at concentrations of less than 4 μM (Fig. 3.10).



**Figure 3.10.** Slow worm DNA (99 ng/μL), *Deroceras reticulatum* DNA (1 ng/μL) and a mixture of the two in the same concentration, amplified in PCR with universal primers LCO1490 (Folmer *et al.* 1994) and C1-N-1777 in the presence of blocking primer SW-1495-C3 at concentrations of 2 μM, 4 μM, 6 μM, 8 μM, 10 μM.

The PCR product amplified from the mixed DNA with 0 μM and 4 μM SW-1495-C3 were each cloned into the pCR 2.1 TOPO TA cloning vector (Version V, Invitrogen), transformed into TOP10 chemically competent *Escherichia coli* (Invitrogen) and positive transformants selected for sequencing by blue/white screening.

Just 4  $\mu\text{M}$  of blocking primer was sufficient to bias PCR amplification 100% in favour of the rare (1%) template (Fig. 3.11).



**Figure 3.11.** Number of clones / sequences belonging to either slow worm or *Deroceras reticulatum* following PCR with universal primers and cloning of an artificial mixture of DNA in a ratio of 99:1; either with 4  $\mu\text{M}$  of blocking primer (SW-1495-C3) or without.

### 3.3 Discussion

The final method demonstrated here (3.2.3) is a fast and cheap means for the complete removal of specific unwanted DNA templates from a mixture, used here to amplify prey DNA from faeces in PCR with universal primers which would typically be dominated by the undigested and abundant predator DNA in the reaction. Previously there has been only one study, by Vestheim and Jarman (2008), using 'PCR clamping' to block predator DNA in dietary analysis. Their study, similarly, used a modified oligonucleotide with a 3' C3 spacer, specific to the predator template. The approach requires no specialised protocol or *a priori* knowledge of potential prey taxa, although the specificity of the method cannot be unequivocally established without extensive empirical testing, a

constraint on any blocking method. Blocking efficiency was related to the amount of blocking primer in the reaction. The optimum concentration for blocking primer was also dependent on the concentration of targeted DNA. In concordance with Vestheim and Jarman's (2008) study, a 10-fold molar excess of blocking primer to universal primers was sufficient to completely block amplification of predator DNA.

Blocking was only successful when overlapping with a universal primer site, preventing annealing. The blocking primer designed to prevent elongation failed to reduce excessive predator amplification, even at a greater than 30-fold molar excess of blocking primer. These results contrasted with Vestheim and Jarman's (2008) attempt at using an elongation arrest primer, which resulted in amplification failure of target and non-target "prey" DNA. They postulated that this was because universal primers were still able to attach to the excess of predator templates even though they could not amplify them, and therefore these primers may not have encountered the rarer prey fragments. In our study, either the blocking primer suffered from inefficient annealing, or *Taq* polymerase was able to extend around / despite it. Von Wintzingerode *et al.* (1997) and Peano *et al.* (2005) both showed that blocking by elongation arrest worked, using PNA, but found that annealing inhibition was more efficient.

Restriction enzyme digest of predator DNA following PCR with universal primers resulted in an insufficiently low yield of prey DNA for successful cloning, corroborating the findings of Blankenship (2005), who used a post-PCR digest of predator DNA in the study of bivalve diet. Dunshea (2005) successfully reduced predator swamping by restriction enzyme digest of faecal DNA preceding PCR, using as little as 5 U of the *PacI* endonuclease. In the current study, pre-PCR digest with predator-specific *StyI* endonuclease failed to visually reduce predator amplification (as determined by presence and brightness of bands produced by gel electrophoresis) even using 64 U.

However, predator DNA was not quantified by cloning, so it is possible that the restriction digest did reduce the amount of predator template.

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## **Chapter 4**

# **Pyrosequencing of prey DNA in reptile faeces reveals benefit of nocturnal hunting**

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# **Pyrosequencing of prey DNA in reptile faeces reveals benefit of nocturnal hunting**

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## 4.1 Abstract

1. Little quantitative ecological information is available on the diets of most reptiles that eat invertebrates, particularly species that are night-active and difficult to observe directly.
2. Few reptiles are night-active and such behaviour is likely to be related to prey availability. Here we addressed the hypothesis that night-hunting may have evolved in slow worms to exploit deeper-living earthworm species, which only come to the surface at night.
3. Although slow worms are widespread and locally abundant across much of Europe, surprisingly little is known about their diet other than that they eat earthworms, slugs and other invertebrates.
4. We used 454 pyrosequencing, an approach new to the analysis of predation in terrestrial ecosystems, to determine which species and ecotypes of earthworm are exploited as prey.
5. Faecal samples from four different habitats were analysed using earthworm-specific primers. Over 86% of slow worms ( $N=80$ ) were found to have eaten earthworm. Numbers of sequences identified through pyrosequencing for each earthworm species provided a measure of the relative biomass consumed.
6. In both lowland heath and marshy / acid grassland *Lumbricus rubellus*, a surface-dwelling epigeic species, dominated the slow worm diet. In two other habitats, riverside pasture and calciferous coarse grassland, the earthworm biomass consumed was dominated by deeper-living anecic and endogeic species.

7. While slugs and epigeal earthworms are probably accessible at any time of day, the deeper-living earthworms could only have been predated at night. We conclude that exploitation of such prey may well have been a significant factor in the evolution of night hunting by these reptiles. All ecotypes are exploited, a fact that helps explain the success of slow worms in both natural and anthropogenically altered habitats. Analysis of prey DNA in reptile faeces by pyrosequencing was found to be a practical, rapid and relatively inexpensive means of obtaining detailed and valuable ecological information on invertebrates in the diet of a reptile.

## 4.2 Introduction

The activity patterns of predatory reptiles are a balance between three major factors: thermoregulation, predator avoidance and prey availability. In warmer climates the first of these is far less of a constraint, if at all (Shine and Madsen 1996), and being nocturnal is common for lizards (notably Gekkonids) and snakes in the tropics (Kearney and Predavec 2000). Reptiles such as geckos are active at night when insect prey are abundant and when the geckos themselves are less vulnerable to predators, particularly birds. In temperate regions, however, most reptiles need to warm up before they become active enough to hunt (Huey and Slatkin 1976). As a result, few have evolved to operate in cool night temperatures and those that have must do so because of the second and third factors, avoidance of predators and the availability of prey.

Slow worms (*Anguis fragilis*) are the most widespread and locally abundant reptile species found in the UK, yet due to their elusive nature they are probably the least understood (Beebee and Griffiths 2000). They are protected under the Countryside and Wildlife Act 1981, requiring developers to relocate populations from sites prior to development, without any guidelines to the suitability of receptor habitats. Furthermore, due to their local abundances, with densities estimated to reach up to 2,100 per hectare (Smith 1990; Riddel 1996; Platenberg 1999), they may play a significant role in epigeal food webs, both as predators and prey. To understand their place in trophic webs it is essential to identify their prey; development of conservation strategies for protected species are often reliant on a sound knowledge of diet (e.g., Marrero *et al.* 2004; Cristóbal-Azkarate and Arroyo-Rodríguez 2007).

Atypical of most temperate reptiles, slow worms are nocturnal / crepuscular hunters (Luiselli 1992). Determining their diet by direct observation is not possible because they not only hunt in the dark but they are semi-fossorial, usually foraging in loose soil and under leaf litter and rocks. Captive experiments on prey preference only tell us what they are capable of eating under artificial conditions with limited prey (Smith 1990; Lavery *et al.* 2004). Records of their diet in the field are largely anecdotal. Their diet in the UK has not been studied before, but in Italy dissections of 24 slow worms killed on roads revealed that, although they were found to have consumed some insects and spiders, their diet was dominated by molluscs and earthworms, with each accounting for approximately a third of the prey items found (35% and 33% respectively) (Luiselli 1992). A study of slow worms in Denmark analysing regurgitates also found molluscs and earthworms to be major prey (44% and 21% respectively) (Pedersen *et al.* 2009). However, the species and diversity of those taxa were not determined, with prey only identified to Order, and with the majority of the ingested remains too damaged even for that level of separation.

Identification of prey by gut content analysis through stomach flushing (Huyghe *et al.* 2007), dissections (Luiselli 1992; Doan 2008) or visual inspection of faecal samples (Amat *et al.* 2008; Clusella-Trullas and Botes 2008; Rodriguez *et al.* 2008) is always limited by its reliance upon the preservation and identification of morphologically distinguishable hard remains (vertebrate bones, otoliths, scales, feathers or arthropod cuticles (Sunderland, Powell and Symondson 2005)). Reptiles frequently feed on soft-bodied prey (Ballinger, Newlin and Newlin 1977; Gunzburger 1999; Pincheira-Donoso 2002), but as these prey are quickly destroyed by digestive processes, leaving no recognisable remains, dietary analyses based on dissection or visual analysis of faeces are prone to biases (Pincheira-Donoso 2002).

Molecular techniques have provided a solution to these constraints through the use of tissue fatty acid signatures (Iverson *et al.* 2004), monoclonal antibodies (reviewed in Greenstone 1996; Symondson and Hemingway 1997; Symondson 2002) or more recently the detection of prey DNA in guts or faeces (reviewed in Symondson 2002; King *et al.* 2008a; Valentini *et al.* 2009b).

The latter approach, using PCR, has been applied successfully to study the diets of a wide range of species. Many studies have designed and applied species-specific primers to analyse, for example, predation on individual species of fish (Parson *et al.* 2005), crustaceans (Vestheim *et al.* 2005; Meekan *et al.* 2009), molluscs (Dodd 2004; Harper *et al.* 2005; Foltan *et al.* 2005) and insects (Agustí *et al.* 2003a,b; Harper *et al.* 2005; Juen and Traugott 2007; Harwood *et al.* 2007). Others have used group-specific PCR primers to analyse predation at a range of taxonomic levels that target, for example, marine vertebrates and invertebrates (Jarman *et al.* 2004; Deagle *et al.* 2005), earthworms and aphids (Harper *et al.* 2006) and Collembola (Kuusk and Agustí 2008). A recent advance has been the application of universal primers, in conjunction with a blocking oligo that limits unwanted amplification of DNA from the predator (Vestheim and Jarman 2008).

The recent development of 'next generation' sequencing technologies such as the Genome Sequencer FLX platform (Roche, Branford, CT, USA) promises to transform the field of DNA-based dietary research, allowing ultra high-throughput DNA sequencing capable of generating 100 million base pairs in 400 base reads per 10 h run (Margulies *et al.* 2005; Wheeler *et al.* 2008). This technology is ideal for dietary analyses which deal with mixtures of prey DNA and relies on detection of the short fragments that survive digestion in the guts of predators. The use of this approach was first predicted to be the method of choice

for the future by King *et al.* (2008a) and within a year the effectiveness of this technique was demonstrated in an analysis of predation by fur seals (Deagle *et al.* 2009) and analyses of the diet of phytophagous species of mammals, birds, insects and molluscs (Soininen *et al.* 2009; Valentini *et al.* 2009a).

All of these PCR-based techniques are useful for studying the diet of vertebrates, for which non-invasive methods are preferred. They are particularly suited to the study of earthworms in the diet of slow worms, a soft-bodied prey that provides no recognizable remains in the faeces of these lizards. In most temperate soils, earthworms represent the single largest macro-invertebrate biomass (Paoletti 1999; Lavelle and Spain 2001). There are over 3500 described species worldwide adapted to a wide range of environments. Many more undoubtedly remain to be discovered (Deleporte 2001; Bohlen 2002; Fragoso *et al.* 1999), with molecular phylogenetics exposing unexpectedly high numbers of cryptic species (King *et al.* 2008b). Given their abundance in the soil and limited defenses it is not surprising that they are a rich source of prey for many species (Lee 1985). Density and diversity of earthworms can vary greatly depending on habitat (e.g. Cesarz *et al.* 2007), influenced by numerous abiotic factors such as temperature, moisture and soil properties (especially pH and organic matter content) (Guild 1948; Edwards and Lofty 1977; Edwards and Bohlen 1996), along with biotic factors such as predation and competition (e.g. Klok and de Roos 1996; Klok 2007). Patterns of earthworm activity vary widely and they occupy a range of soil depths dependent upon species, season and life history stage (Bouche and Gardner 1984) and densities may vary from less than a single individual per m<sup>2</sup> to several hundred (Curry 1998; Lee and Plankhurst 1992). Therefore the presence or absence of

appropriate habitat for earthworms may indirectly influence the suitability of a site for their predators.

Here our aim was to test the hypothesis that slow worms are crepuscular and nocturnal hunters because it is only at these time that many of the deeper-living (and larger) earthworm species come to the surface (to pull leaves down into their burrows (Duriez *et al.* 2006)). To do this we analysed the spectrum of earthworm species in slow worm diets in four different habitats (lowland heath, marshy/acid grassland, coarse grassland and pasture) with their different earthworm assemblages. Although earthworms are known to be a major prey of slow worms almost nothing is know about the species consumed (Luiselli 1992). Only molluscs have been found to be similarly abundant in their diet, based on dissections (Luiselli 1992) and regurgitates (Pedersen *et al.* 2009), but the generally larger size of earthworms, and greater densities, means they are probably more important in terms of prey biomass. This is the first time that pyrosequencing has been used to study the invertebrate diet of any predator in a terrestrial system and the first use of any molecular technique to analyse prey DNA in reptile faeces. A key aim was to establish whether prey DNA could be detected and identified from reptile faeces. If it could then this approach would have the potential for much wider application to future studies of reptile ecology and trophic relationships. Another more specific aim was to establish whether particular earthworm functional groups dominate the diet of slow worms: whether they are restricted to feeding primarily on more accessible surface dwelling epigeic earthworm species, or whether they are able to access a broader range of prey resources that includes the larger deeper living earthworm species. Should they be able to do so this would provide them with a potentially

abundant source of nutrition, only available at night and denied to most other reptiles and diurnal competitors.

### 4.3 Methods

#### *Primer testing and captive feeding trial*

Earthworm group-specific mtDNA primers 185F (5'-TGTGTAAGCGCCGTCGTAAGCA-3') and 14233R (5'-AAGAGCGACGGGCGATGTGT-3') (Harper *et al.* 2005), which target a 225-236 bp fragment of the 12S gene, were optimised for specificity in PCR using DNA from slow

	<b>Species</b>	<b>Family</b>	<b>Order</b>
<b>Targets</b>	<i>Lumbricus terrestris</i>	Lumbricidae	Haplotaxida (earthworms)
	<i>L. rubellus</i>	Lumbricidae	Haplotaxida (earthworms)
	<i>Aporrectodea longa</i>	Lumbricidae	Haplotaxida (earthworms)
	<i>A. caliginosa</i>	Lumbricidae	Haplotaxida (earthworms)
	<i>Allolobophora chlorotica</i>	Lumbricidae	Haplotaxida (earthworms)
<b>Non-targets</b>	<i>Arion intermedius</i>	Arionidae	Pulmonata (slugs)
	<i>A. owenii</i>	Arionidae	Pulmonata (slugs)
	<i>A. hortensis</i>	Arionidae	Pulmonata (slugs)
	<i>A. ater</i>	Arionidae	Pulmonata (slugs)
	<i>A. distinctus</i>	Arionidae	Pulmonata (slugs)
	<i>Deroceras reticulatum</i>	Agriolimacidae	Pulmonata (slugs)
	<i>Limax maximus</i>	Limacidae	Pulmonata (slugs)
	<i>L. flavus</i>	Limacidae	Pulmonata (slugs)
	<i>Erigone dentipalpis</i>	Linyphiidae	Araneae (spiders)
	<i>Adalia bipunctata</i>	Coccinellidae	Coleoptera (beetles)
	<i>Pteristichus niger</i>	Carabidae	Coleoptera (beetles)
	<i>Forficulidae sp.</i>	Forficulidae	Dermaptera (earwigs)
	<i>Scaeva pyrausti</i>	Syrphidae	Diptera (true flies)
	<i>Metasyrphus luniger</i>	Syrphidae	Diptera (true flies)
	<i>Tipulidae sp.</i>	Tipulidae	Diptera (true flies)
	<i>Aphis fabae</i>	Aphidae	Hemiptera (true bugs)
	<i>Cercopidae sp.</i>	Cercopidae	Hemiptera (true bugs)
	<i>Galleria mellonella</i>	Pyralidae	Lepidoptera
	<i>Anguis fragilis</i>	Anguidae	Squamata

**Table 4.1.** Target and non-target taxa tested for specificity with 185F and 14233R 12S primers earthworm group-specific primer.

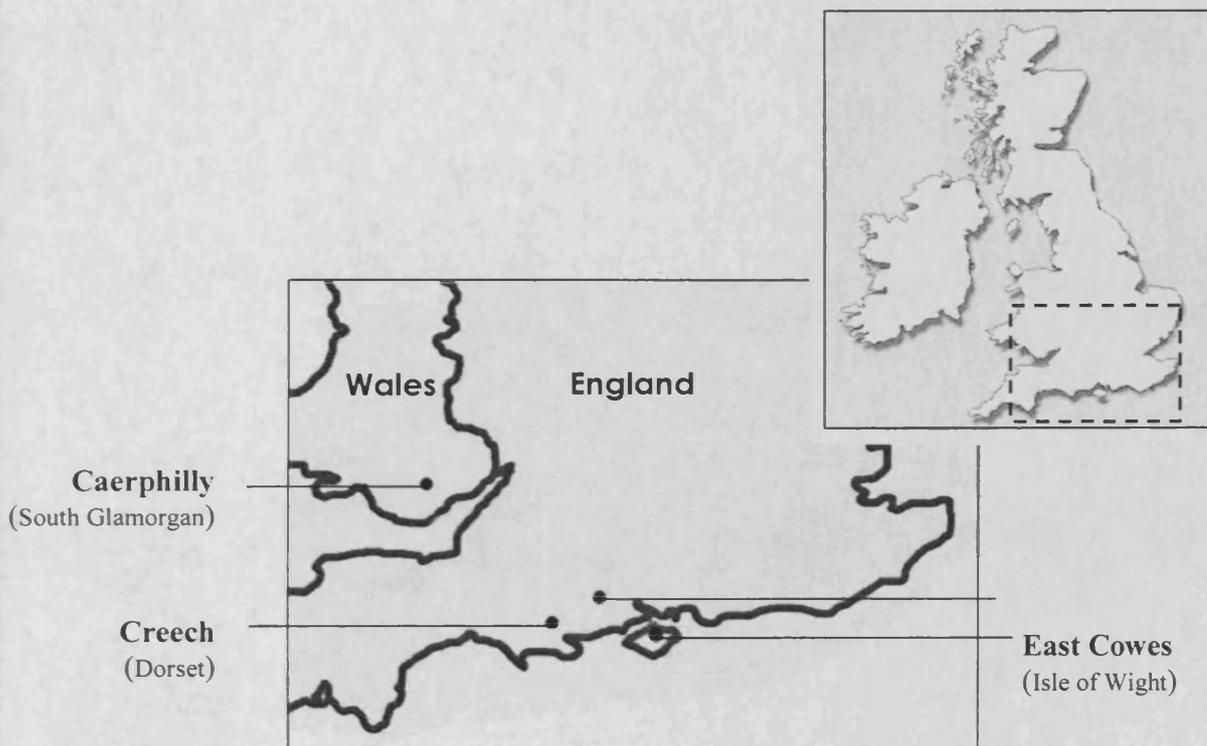
worms and 18 different invertebrate species representing a wide range of taxa (Table 4.1). The advantage of using these particular primers was that there was complete homology at the primer sites of all species examined, ensuring equal amplification success. Optimised conditions were: 1× buffer, 2 mM MgCl<sub>2</sub>, 0.5 mM dNTPs (Invitrogen), 0.5 μM of each primer, 0.375 U *Taq* polymerase (Invitrogen) and 1.5 μL/25 μL of template DNA with an initial denaturation at 94°C for 3 min, and 35 cycles of 94 °C for 30 s, 65 °C for 45 s, 72 °C for 1 min with a final extension at 72 °C for 2 min.

Before field work commenced, a laboratory study was conducted to ensure that amplifiable prey DNA could be obtained from the faeces of these reptiles, something that had never been attempted before. Twenty adult slow worms were maintained in a controlled environment room at 16°C on a L:D regime of 16:8 h, in separate plastic tanks. After a minimum of two weeks on an exclusive diet of slugs (*Deroceras reticulatum* and *Arion* spp.) faecal samples were collected and DNA extracted using the QIAamp® DNA Stool Mini Kit (Qiagen) in accordance with the manufacturer's instructions. Slow worms were then maintained on a diet of earthworms (*Lumbricus terrestris*) for two weeks and DNA extracted from faeces. All faecal DNA was screened in PCR with the earthworm group-specific primers 185F and 14233R using the conditions described above, including water negative controls to exclude the risk of contamination.

#### 4.3.1 *Field study*

Faecal samples were collected from adult slow worms in July 2007 at four locations, chosen for their contrasting habitats. There were totals of 21 samples from Caerphilly (South Glamorgan), 19 from Flat Holm (South Glamorgan), 19 from Ringwood (Hampshire) and 21

from East Cowes (Isle of Wight) (see Fig. 4.1). The Caerphilly site (51°22'N, 3°13'W) is an approximately five hectare area of marshy grassland, comprising purple moor grass (*Molinia*



**Figure 4.1.** Locations of sampling sites where slow worm faecal samples were collected.

*caerulea*), tufted hair grass (*Deschampsia cespitosa*), gorse (*Ulex* spp.), bramble (*Rubus fruticosus*), various fern species (*Dryopteris* spp.), scattered birch trees (*Betula* spp.) and numerous associated pond flora, surrounded by areas of species-poor acid grassland. Flat Holm (51°22'N, 3°07'W), in the Bristol Channel, is a 24 hectare island dominated by coarse grass species such as false oat grass (*Arrhenatherum elatius*), with patches of species-rich red fescue (*Festuca rubra*) dominated maritime grassland occurring close to the cliffs and an

absence of any mature trees. The Ringwood site (50°52'N, 1°51'W) is an approximately four hectare area of Ericaceous heathland consisting of common heather (*Calluna vulgaris*), bell heather (*Erica cinerea*) and gorse (*Ulex* spp.) surrounded by coniferous woodland and adjacent to an area of unimproved grassland. The East Cowes site is an abandoned riverside pasture, dominated by grasses and some ferns (*Dryopteris* spp.).

Faecal samples were collected into 1.5 ml Eppendorf tubes by gentle palpation of the slow worms. Faeces were collected from approximately equal numbers of males and females at each location. Prey DNA was extracted from faeces using the QIAamp® DNA Stool Mini Kit (Qiagen) in accordance with the manufacturer's instructions.

#### 4.3.2 Earthworm group-specific primers

DNA extraction success of samples was determined by PCR amplification with the general cytochrome oxidase I (COI) primers LCO1490 (Folmer *et al.* 1994) and HCO1777 (5'-ACTTATATTGTTTATACGAGGGAA-3'), a primer designed to be general and found to successfully amplify a wide range of taxa (Arthropoda, Annelida, Mollusca and Chordata). These primers amplify a 287 base pair amplicon in both invertebrates and the slow worm predator and were a control for the presence of amplifiable (but not necessarily prey) DNA (King *et al.* 2008). Conditions were: 1× buffer, 2 mM MgCl<sub>2</sub>, 0.1 mM dNTPs (Invitrogen), 0.5 μM of each primer, 0.45 U *Taq* polymerase (Invitrogen) and 1 μL/25 μL of DNA with an initial denaturation at 94°C for 3 min, 45 cycles of 94 °C for 30 s, 46 °C for 1 min and 72 °C for 1 min, and a final extension at 72 °C for 5 min. Amplification success was visualized by gel electrophoresis. With the presence of DNA confirmed and extraction success established, prey DNA was amplified in PCR with the earthworm-specific 12S primers 185F

and 14233R (Harper *et al.* 2005) with the fusion primers necessary for GS-FLX sequencing (5'-GCCTCCCTCGCGCCATCAG-3' and 5'-GCCTTGCCAGCCCGCTCAG-3' respectively) attached to the 5' ends. Conditions used were as above (see Primer testing). Presence / absence of earthworm DNA was determined by gel electrophoresis visualized with ethidium bromide. DNA concentration of PCR products was quantified using the Nanodrop ND-1000 spectrophotometer, and equal concentrations pooled from all slow worm faecal samples from each of the four locations, prior to pyrosequencing on the GS-FLX platform.

#### 4.3.3 *Data analysis*

The pooled samples for each of the four locations were sent to Macrogen for sequencing using the GS-FLX (Roche) platform. DNA mixtures from other experiments were included in the same segments on the PictoTitre Plate and run at the same time, greatly reducing the costs. Different primers were used and genes targeted in the different experiments, making it easy to assign returned sequences to the correct experiment. Sequence sorting and clustering was executed by purpose-written software as described in Deagle *et al.* (2009). Sequence reads without an exact match to one of the PCR primers, or that were below 60 bp, were discarded. The remaining sequences for each of the four field locations were grouped by similarity to each other by making a pairwise alignment with ClustalW (Thompson *et al.* 1994), with gap opening and extension parameters of 10 and 0.5 respectively, and then calculating the LogDet DNA distance (Lockhart *et al.* 1994) amongst all sequences. Sequences were grouped into mutually exclusive clusters of sequences with not more than 0.05 LogDet units difference amongst them. The sequences within groups were aligned and visually checked for distinct sequences to make sure that multiple species were not grouped

within the 0.05 LogDet threshold. Sequences representative of each cluster were compared with the GenBank database by BLAST searching (Altschul *et al.* 1990), in addition to comparison with in-house DNA libraries. Sequences with  $\leq 95\%$  match were considered as unidentified.

#### 4.4 Results

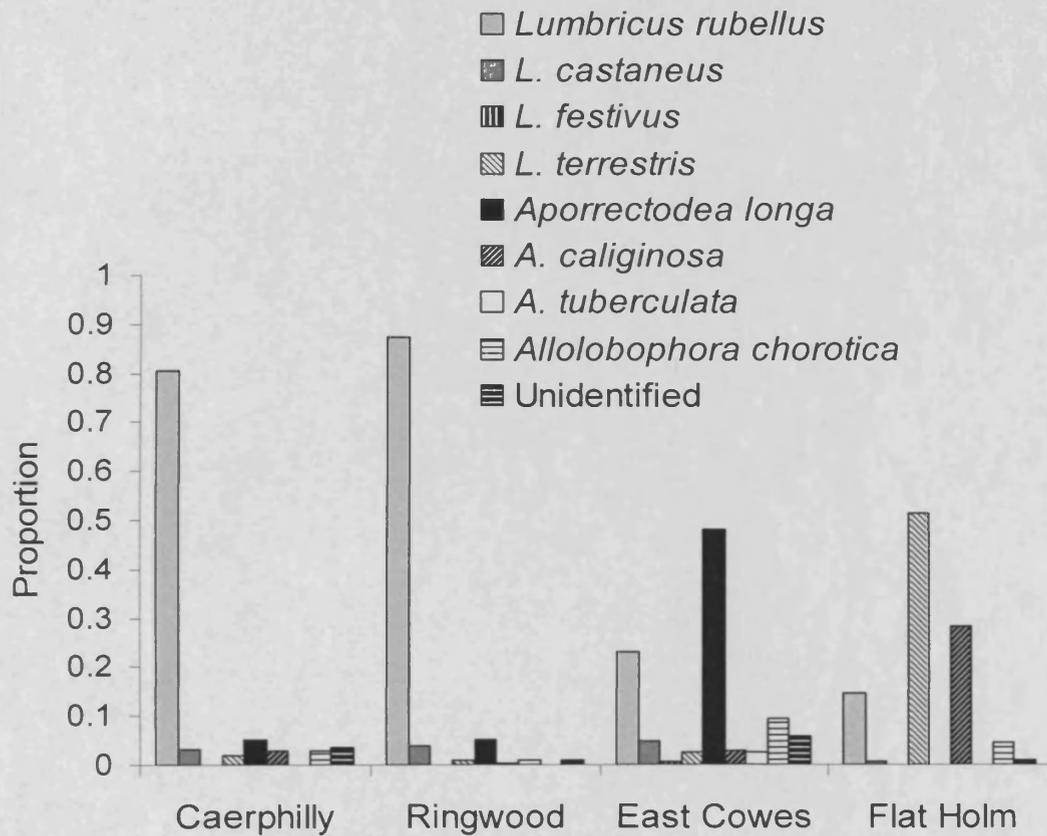
The earthworm group-specific primers successfully co-amplified all of the earthworm species tested (Table 4.1) but (as intended) none of the non-earthworm species or the slow worms themselves. These cross-amplification tests were in addition to those performed previously with these primers by Harper *et al.* (2005), which also showed specificity to earthworms.

Faecal samples from all 20 captive slow worms fed on a diet of *L. terrestris* tested positive for earthworm using the earthworm-specific primers. This proved beyond doubt that prey DNA could be detected in slow worm faeces. No faecal samples from control slow worms fed, in parallel, on slugs tested positive, confirming that positives represented detection of earthworm consumption.

Of the faecal samples collected from wild slow worms, and screened with the earthworm group-specific 12S primers, 91% of those from Caerphilly, 79% of those from Ringwood, 100% of those from East Cowes and 71% of those from Flat Holm tested positive for the presence of earthworm DNA. These results confirmed that earthworms in general were a frequently-consumed component of the diet of slow worms in the wild.

Of the 1685 sequences returned only 48 (2.9%) could not be identified with confidence, representing either sequencing errors or species for which sequences were not

available for comparison. The proportions of sequences from different earthworm species identified during pyrosequencing provides a semi-quantitative measure of the biomass of each species detected in the samples (Deagle *et al.* 2009). The proportions found in the pooled DNA extracted from the faecal samples from each of the four field sites are shown in Fig. 4.2. Excluding *Lumbricus festivus* and *Aporrectodea tuberculata* from analysis, due to the low number of sequences obtained for them, there was a significant difference in the numbers of sequences for each earthworm species between locations ( $\text{Chi}^2=1605$ ,  $\text{DF}=15$ ,



**Figure 4.2.** Proportions of earthworm prey DNA sequences, extracted from slow worm faeces and detected by pyrosequencing, from four locations: Caerphilly (total sequences  $n = 624$ ), Ringwood ( $n = 473$ ), East Cowes ( $n = 300$ ) and Flat Holm ( $n = 318$ ).

$P < 0.001$ ). At the two mainland sites, Caerphilly and Ringwood, the diet was dominated by the surface-living *L. rubellus*, with little predation indicated on other species. However, at the two island sites, East Cowes and Flat Holm, the most frequently consumed earthworms were the deeper-living species *L. terrestris* and *A. longa*, although the proportions of sequences suggest a more varied diet.

#### 4.5 Discussion

Screening of slow worm faecal DNA with earthworm primers was found to be 100% effective in the captive study. This was by no means a forgone conclusion. Many reptiles are known to digest their prey extremely thoroughly, dissolving bones and other hard parts (Secor 2008). Nobody has previously attempted to extract prey DNA from reptile faeces. The only other study using molecular analysis to determine a component in the diet of a reptile, of which we are aware, is that by Nelson *et al.* (2000), in which they were able to identify a partially-digested Cape Sable seaside sparrow found in the gut of a dead snake (*Agkistrodon piscivorus*). The potential for future work on reptiles is therefore considerable. Although slow worms may not be typical, and the digestive systems of other reptiles may be more efficient, we have shown in other work (in the laboratory) that the technique works equally well in five species of snake (unpublished data).

When applied to slow worms in the field, earthworms were found in the diet of a majority of slow worms at all four locations, ranging from 71% on Flat Holm to 100% at East Cowes, confirming the significance of these invertebrates as prey. Previous non-molecular work on slow worms has shown that earthworms made up about a third of prey items in Italy (Luiselli 1992) and a fifth of prey items in Denmark (Pedersen *et al.* 2009) and

it may be that in the wetter conditions in the UK annelids are a more important prey item. No previous study, however, has examined the species of earthworm being consumed in the field, nor compared dietary choices between sites.

Given the diversity of earthworm species likely to be present, in such different and heterogeneous habitats, application of a panel of species-specific primers was not considered practical. Such multi-primer analyses are in any case problematic, in that each primer pair will target different primer sites and therefore the primers will have different abilities to detect semi-digested prey DNA, making comparison between rates of predation on different species difficult. With invertebrate predators, feeding trials can be conducted and median detection periods established, which can be used to calibrate, and correct for, differences in survival of target amplicons during digestion (King *et al.* 2008a). Such an approach is impractical for vertebrates, where housing sufficient numbers for valid sample sizes is not possible and, furthermore, is inappropriate for faecal analysis, where the period from prey ingestion to faecal sample collection cannot be determined or standardised.

To date only a few studies have applied pyrosequencing to dietary analysis (Deagle *et al.* 2009; Valentini *et al.* 2009a; Soininen *et al.* 2009), with only that of Deagle *et al.* (2009) looking at carnivory. They found this to be an efficient alternative to cloning and sequencing in identifying a broad range of prey from predator faeces (Deagle *et al.* 2009). In order to provide a quantitative estimate of diet from the sequence libraries that arise from pyrosequencing, the proportion of DNA recovered from faeces for any given prey species must approximately reflect the proportional biomass of that prey ingested, which has been suggested, in some systems at least, to be the case (Deagle and Tollit 2007). The validity of such quantitative estimates is greatly improved by using a single primer pair with complete

homology at the primer site, ensuring (in this case) that both primer efficiency and target amplicon size are invariable. Deagle *et al.* (2009) discuss a number of complicating factors which may cause amplification bias. Firstly, there may be variation in the copy number of mitochondrial genes in cells from different tissues and different species (Prokopowich *et al.* 2003). Additionally, the susceptibility of different tissues / species to digestive processes may vary. Finally, primer mismatching in some species may result in differences in amplification efficiency between prey (e.g. Polz and Cavanaugh 1998; Deagle *et al.* 2007). In our study, these potentially confounding factors are unlikely as all prey belonged to the same family, whereas Deagle *et al.* (2009) were comparing amongst species belonging to several phyla. A single group-specific primer pair was used, to address specific hypotheses related to earthworm consumption only, and therefore there is no reason to expect species bias in PCR. We consider the results presented are a relatively accurate measure of the proportions of different earthworm species consumed, in terms of biomass.

There were significant differences in the earthworm component of slow worm diet between the four habitats (see Fig. 2) ( $P < 0.001$ ). Earthworm species richness in the diet ranged from 6-9 across the four locations, with the lowest diversity found in the lowland heath at Ringwood and the highest in the pasture in East Cowes. Differences in diet from the four locations probably reflected differences in prey availability rather than any geographical differences in prey preference. The presence of ecologically different earthworm groups (epigeic, anecic and endogeic) in their diet suggests slow worms are opportunistic predators; however, as earthworm diversity and abundance were not measured at the sites, conclusions about prey choice cannot be made. Ideally, one would compare the proportions of different prey species available with those detected in the faecal samples. We know from previous

work with invertebrate predators that prey choice (incorporating not simply preferences but also factors such as encounter rates and prey escape strategies) can be profound and that different prey species are rarely consumed in the proportions available (cf. Agustí *et al.* 2003b; King *et al.* 2010). The studies by Agustí *et al.* (2003b) and King *et al.* (2010) were in relatively uniform arable crops. However, such an approach is fraught with difficulties and probably meaningless in heterogeneous natural habitats. All earthworm sampling methods are prone to biases. Hand-sorting is biased towards adults and larger earthworms (Edwards 1991), formalin extraction is toxic to worms (Lee 1985; Gunn 1992) and biased towards deep-living species which form vertical burrows (Raw 1960), and mustard extraction underestimates deep-living species (Lawrence and Bowers 2002; Bartlett *et al.* 2006) and is inappropriate for accurate assessment of earthworm assemblages (Bartlett *et al.* 2006). Juveniles meanwhile cannot be identified, unless each is separately barcoded. By far the greatest problem however is that the slow worms were free to move between, and exploit, a variety of sub-habitats within heterogeneous landscapes (containing different earthworm assemblages) and any assessment of prey in one area would not accurately reflect a predator's utilisation of it.

At two of the sites, Caerphilly and Ringwood, *L. rubellus* dominated slow worm diets, accounting for 80% and nearly 90% of earthworm sequences, respectively. At East Cowes and on Flat Holm, however, it accounted for only 23% and 15%. This indicates that there was probably a much greater availability of *L. rubellus* at the Caerphilly and Ringwood sites. *Lumbricus rubellus* is one of the most acid-tolerant earthworm species, found in soils with a pH as low as 3 (Curry 1998), and it may be this tolerance that explains its dominance at these acidic sites. Earthworms are generally absent from sandy soils, preferring instead

medium-textured loamy soils (Guild 1948; Cotton and Curry 1980; Edwards 1996). Cotton and Curry (1980), for example, found a seven-fold higher density of earthworms in loamy grassland compared to a similarly managed sandy grassland. Most earthworm species are intolerant of soil pH below 4.5 (Satchell 1967; Bouche 1972). The heathland site in Ringwood is situated adjacent to an area of unimproved grassland and areas of coniferous woodland offering more favourable conditions for earthworms (Lee 1985). As slow worms have home ranges up to nearly 800 m<sup>2</sup> (average: 200 m<sup>2</sup>) (Smith 1990), it is possible that slow worms were foraging in these more earthworm-friendly peripheral areas too, which may explain the presence of a diversity of earthworm species in their diet in this unfavourable habitat, but with most species being detected at low relative abundances.

Alternatively, *L. rubellus*, a surface-living earthworm species, is likely to be encountered more frequently by slow worms than anecic and endogeic species, and the slow worm diets of Caerphilly and Ringwood may reflect this higher encounter rate. Slow worm diet in the pasture in East Cowes was dominated by *Aporrectodea longa*, a species frequently found to be dominant in such habitat (Guild 1951). While the habitats of East Cowes and Flat Holm are suitable for *L. rubellus* its far smaller proportion of slow worm diet compared to the other sites may reflect a lower density. This may be because these ecosystems are less structurally complex and more open, offering less protection to surface-dwelling earthworms from predation by hedgehogs, badgers, shrews, moles and, above all, birds. This is particularly likely on Flat Holm, which has significant breeding colonies of over 4,000 pairs of Lesser Black-backed gulls (*Larus fuscus*) and 400 pairs of Herring gulls (*L. argentatus*) amongst others. On these sites slow worms are consuming more endogeic and anecic species which come to the surface at night.

The results revealed that slow worms are not limited to feeding on epigeic species, but can access anecic and endogeic species too, which normally only come to the surface at night. Consumption of such deeper-living species supports the hypothesis that the slow worms may have made this adaptation to nocturnal and crepuscular hunting in order to access otherwise unobtainable prey, and to avoid competition for prey with diurnal predators of earthworms (particularly birds). This behaviour has the added benefit of reducing a reptile's risk of predation, from snakes and birds for instance, allowing them to survive in areas of high predator densities such as amidst the sea bird colonies of Flat Holm. In terms of management and translocation programs, it appears that while the presence of an abundant supply of earthworms is important, the species and ecological group to which they belong is not. In a wider context we can conclude that analysis of prey DNA in reptile faeces is a practical means of obtaining precise information on diet. When combined with pyrosequencing, such analyses provide a powerful means of rapidly, and relatively inexpensively, obtaining valuable ecological information about the diets of highly generalist predators.

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## **Chapter 5**

**Seasonal and sex-related differences**

**in the diet of slow worms.**

## 5.1 Abstract

Slow worms (*Anguis fragilis*) are secretive, nocturnal and semi-fossorial reptiles, making it impossible to establish their diet through direct observation in the field. Previous knowledge of their diet is, therefore, based mostly on captive studies and a couple of European studies which revealed them to mostly be consuming earthworms and pulmonates. A shortcoming of these studies is that they relied on the visual identification of prey in faeces, regurgitates and gut dissections and as such were not able to determine most prey to species or even family level. Molecular methods overcome these biases and constraints. Using prey species-specific PCR primers for a range of pulmonates and earthworms we analysed faeces from 400 slow worms collected each month over two years from three sites. We were able to investigate predation on individual prey species, along with exploring ontogenetic, seasonal and sex-based patterns of predation.

Predation by slow worms on pulmonates and earthworms was much higher than reported in previous studies, with 50% and 65% of slow worms found to have consumed them, respectively. However, no ontogenetic differences were found in relation to their predation, indicating that slow worms consume the same prey throughout their lifetime. There were distinct seasonal patterns of predation on individual prey species, probably reflecting fluctuations in prey availability. Predation on certain slugs and earthworms was influenced by weather (rainfall and temperature), factors known to influence surface activity of these prey. This, in conjunction with seasonal patterns of predation, suggests that slow worms are opportunistic in their consumption of these prey. Predation on *Arion* spp. slugs by females was higher in spring and autumn than by males, suggesting preferential selection by females, possibly indicating differences in reproductive costs.

These results provide a much clearer picture of slow worm diets and will help to inform future translocation programmes.

## 5.2 Introduction

Diet frequently changes throughout a predator's lifetime, with the type and amount of prey taken often differing seasonally and ontogenetically (Castilla *et al.* 1991; Preest 1994; Teixeira-Filho *et al.* 2003; Herrel *et al.* 2006). Seasonal variation in diet is often caused by fluctuations in prey availability (e.g. Slip and Shine 1988; Houston and Shine 1993; Teixeira-Filho *et al.* 2003; Herrel *et al.* 2006), whereas ontogenetic changes are commonly a result of improved foraging (the ability to handle or catch prey) (Rutz, Whittingham and Newton 2006) or the range of prey available (size and species) increasing with predator size (Dickman 1988). In addition to these, a predator's use of habitat might change both seasonally and ontogenetically (e.g. Shine 1986b) with a corresponding change in access to prey.

Diet can also differ between sexes, due to different morphology (reviewed in Shine 1989) and behaviour (e.g. Pyke, Pulliam and Charnov 1977; Ryan, Bartholomew and Rand 1983; Savitsky 1983). For instance, sexual dimorphism in body size or mouth parts may force males and females to take different sizes, species or amounts of prey. Additionally, differences in nutritional requirements associated with reproduction can lead to differences in diet, or additional dietary components, in females compared to males (e.g. Clutton-Brock, Guinness and Albon 1982; Harrison 1983).

Slow worms (*Anguis fragilis*) are a secretive and semi-fossorial reptile, therefore establishing their diet through direct observation in the wild is not possible. Current understanding of their diet has come from captive studies (Poivre 1972, 1975; Billings 1987; Smith 1990; Lavery *et al.* 2004), which only indicate prey choice in an artificial system, along with limited analyses of faeces, regurgitates or dissections of wild slow worms

(Luiselli 1992; Pedersen, Jensen and Toft 2009). Earthworms, slugs and snails are the most commonly found prey of slow worms (Schreiber 1912; Rollinat 1934; Smith 1964; Petzold 1971; Smith 1990; Luiselli 1992; Luiselli *et al.* 1994; Blosat 1997; Capizzi *et al.* 1998). Luiselli (1992) calculated that slow worms have a low dietary niche breadth and suggested there were relatively restricted dietary specialists. Gut dissections of 24 slow worms in Italy revealed that pulmonates and earthworms each made up around a third of the prey items consumed (33% and 35% respectively) (Luiselli 1992), with diptera, lepidoptera (larvae), coleoptera (larvae and imagines), homoptera and araneidae all constituting less than 10% each. A study of 84 slow worms in Denmark, which involved collecting animals and cooling them at 8°C to induce regurgitation (Pedersen, Jensen and Toft 2009), also found pulmonates (44%) and earthworms (21%) to be predominant prey in the diet, although millipedes, specifically *Glomeris marginata* (22%), were even more common than earthworms. Lepidoptera larvae were consumed in greater numbers (10%) than any other larvae, despite the higher densities of coleoptera larvae present in the field. In their study, millipedes and larvae were predated significantly more at the start of the season (May/June), whereas no seasonal difference in predation on earthworms or pulmonates was detected, although there was an almost significant trend ( $P=0.07$ ) for an increased predation on slugs and snails in September. The seasonal patterns of predation on earthworms corresponded with the density of those prey in the field, implying they were being taken in accordance with their availability. Availability of millipedes, however, increased from spring to autumn, although consumption of them was significantly higher in the spring / early summer, suggesting preferential selection for them at this time. There was no ontogenetic effect on diet

(comparing juveniles with adults) and no difference was detected between males and females on the predation of any prey (Pedersen, Jensen and Toft 2009).

These studies suffer from a dependency on the identification of morphologically distinguishable prey remains in faeces or regurgitates, which is prone to biases (Pincheira-Donoso 2002). This is particularly true with regards to soft-bodied prey, such as earthworms and slugs, which leave few hard recognisable parts. As a result, most prey in these studies could not be identified to species level but only to that of order or family. The detection of prey DNA in the guts, faeces and regurgitates of predators (reviewed in Symondson 2002; King *et al.* 2008a) has been successfully applied to investigating predation on and by a wide range of both invertebrate and vertebrate taxa (e.g. Saitoh *et al.* 2003; Jarman *et al.* 2004; Deagle *et al.* 2005; Harper *et al.* 2005; Redd *et al.* 2008; King *et al.* 2010).

Earthworms represent, by far, the largest biomass of macro-invertebrates in temperate soils (Paoletti 1999; Lavelle and Spain 2001), and due to their abundance and limited defenses they are an important prey for many species (Lee 1985). Different ecological groups of earthworms occupy different soil depths, with epigeic species predominantly surface-dwelling and endogeic species living deep in the soil, only surfacing to pull down leaves into their burrows (Duriez, Ferrand and Binet 2006). The density of different earthworm species is influenced by numerous abiotic factors, including temperature and moisture (Guild 1948; Edwards and Lofty 1977; Edwards and Bohlen 1996), which may vary between habitat and seasonally within habitats from changing weather conditions. Slugs and snails are often abundant taxa and a common prey to many species. One of the most common in the UK is the field slug, *Deroceras reticulatum*, with its high densities partly due to its ability to remain active at low temperatures (South 1992) and to reproduce throughout

the year (Hunter and Symonds 1971). The *Arion* genus comprises several common slug species, including the most common, the garden slug, *Arion hortensis* aggregate. The activity and density of pulmonates, like earthworms, is strongly affected by temperature and rainfall (Rollo 1982; Cook and Ford 1989; Young and Port 1989). While their abundance makes them an obvious prey, the mucus defenses of slugs and hard shells of most snails deters many predators (e.g. Pakarinen 1994; Mair and Port 2002). Slow worms, however, apparently have little or no difficulty with their defenses and are voracious predators of slugs (e.g. Howes 1986) and are known to consume some snails also (e.g. Pedersen, Jensen and Toft 2009).

While pyrosequencing (see Chapter 4) revealed predation on earthworms was diverse and not limited to epigeic species, it gave no indication of what influences predation on individual species. Here we used molecular faecal analyses to investigate the seasonal, ontogenetic and sex patterns of predation by UK populations of slow worms on individual pulmonate and earthworm species. The key aim was to identify factors affecting predation, more specifically to address the null hypotheses: There will be no change in diet with increasing slow worm size (ontogenetic differences); There will be no seasonal variation in predation on slugs, snails or earthworms; There will be no sex differences in the diet of slow worms.

## 5.3 Methods

### 5.3.1 *Field sites and faecal DNA extraction*

Details of the field sites (Caerphilly, Ringwood and Wareham) used in this study, along with details of faecal collection methodology, are described in Section 2.6.1. Faecal samples from

400 slow worm were collected during monthly visits between April and September, 2007 and 2008, to each site.

DNA was extracted from each faecal samples using the QIAamp® DNA Stool Mini Kit (Qiagen), following the manufacturer's instructions. To ensure extraction success, all DNA was amplified in PCR with universal primers LCO1498 (Folmer *et al.* 1994) and C1-N-1777 with the following conditions: 1X buffer, 2 mM MgCl<sub>2</sub>, 0.5 mM dNTP (Invitrogen), 0.5 μM of each primer, 0.38 U *Taq* polymerase (Invitrogen) and 2 μL/ 25 μL of DNA with an initial denaturation at 94 °C for 3 min, 35 cycles of 94 °C for 30 s, 48 °C for 30 s and 72 °C for 45 s, and a final extension at 72 °C for 5 min. Amplification was visualized by gel electrophoresis stained with ethidium bromide. Double-distilled water was included as a negative control to test for contamination.

### 5.3.2 *Multiplex Optimisation*

Species-specific primers (described in Table 5.1) were tested for specificity in various combinations on target DNA in Multiplex using 1X Multiplex PCR Master Mix (Qiagen), 0.2 μM of each primer and 5X bovine serum albumin (BSA), with a PCR cycle of 95 °C for 15 min, and 35 cycles of 94 °C for 30 s, a gradient of 50-65 °C for 90 s, 72 °C for 90 s, and a final extension of 72 °C for 10 min. Amplification was visualized by gel electrophoresis stained with ethidium bromide, and double-distilled water was included as a negative control to test for contamination. Four Multiplexes were selected (A-D, Table 5.1) which produced clear bands for all targets when DNA was mixed, and which produced fragment sizes distinguishable from each other by gel electrophoresis.

SPECIES	MULTIPLEX	GENE	FORWARD PRIMER	REVERSE PRIMER	SIZE	
<i>Lumbricus terrestris</i>	A	COII	COII-Lt-F2 GAACTATTTTCYACATTTAAGAA	COII-Lt-R2 CGGCTATGCTCTYCTAGCAC	256	*
<i>L. castaneus</i>	A	COI	COI-Lc-F2 AACTGACTCCTCCCCTAAT	COI-Lc-R2 AGAAGGTCCTGCGTGAGCT	189	*
<i>L. rubellus</i>	A	COII	COII-Lr-F3 AGACGGTAATCTCCTGGAAGT	COII-Lr-R2 CTTCGTATTCTCTATATCACA	164	*
<i>Aporrectodea longa</i>	A	COI	COI-AI-F2 TGGCTTCTACCTCTAATACT	COI-AI-R2 ATGAAGGGAGAAGATGGCCA	213	*
<i>A. rosea</i> (lineage 1)	B	COI	COI-ArL1-F2 CCTTAATTCTTCTGGTCTCA	COI-ArL1-R2 GTTAAGGGCTCCTAGAATCG	167	*
<i>A. rosea</i> (lineage 2)	B	COI	COI-ArL2-F2 CCATTAACCTTTATTACAACATC	COI-ArL2-R2 ATGTGTTGAGATTTCCGGTCC	171	*
<i>A. caliginosa</i>	C	16S	16S-Ac-F1 CTAAATTCTGACCCTTATTC	WORM-16S-R1 CCTAAGCCAACATCGAGGTG	116	*
<i>Allolobophora chlorotica</i> (lineage 1)	B	COI	COI-AchL1-F4 AAATTGATTACTACCYCTG	COI-AchL1-R2 GAAGCACCTGCTAGRTGG	231	*
<i>Allolobophora chlorotica</i> (lineage 2)	B	COI	COI-AchL2B-F3 CATCACTAATCCTTCTAGTG	COI-AchL2B-R3 AGAAGATAGCTAAGTCTACG	126	*
<i>Allolobophora chlorotica</i> (lineage 3)	B	COI	COI-AchL3-F2 TGGAATTGACTATTACCAC	COI-AchL3-R2 ATGAAATTAATTGCCCGAG	261	*
<i>Helix aspersa</i>	C		Hel-Asp-F	Hel-Asp-R		
<i>Deroceras reticulatum</i>	D	16S	Dr11F CTATACACAATTTTTAAATAAG	DRF29RC GCTTCTGGTTTATCTATTATTTGGT	109	†
<i>Arion</i> spp.	D	12S	Ai1F CACATAAATGATAGTCACC	AR2R ATACCTACAAGTCCATCTTT	208-221	†

**Table 5.1.** Species-specific primers used in four Multiplexes (A-D). \* King *et al.* 2010. † Harper *et al.* 2005.

### 5.3.3. *Primer screening*

All faecal samples were screened with each primer pair twice, and any bands of the correct size determined by gel electrophoresis scored as a positive. Tissue DNA of all earthworm species targeted in that particular Multiplex was included as a positive control, to ensure PCR success, and water was included as a negative control to check for contamination.

### 5.3.4 *Statistics*

The effects of slow worm length, weight and sex, along with site, month, temperature, rainfall and sunshine on predation of prey were explored within a stepwise Generalised Linear Model (GLM). Temperature, rainfall and sunshine data downloaded from the Meteorological Office. Weight, length, temperature, rainfall and sunshine were treated as covariates and all other predictors as factors. A binomial error distribution was used with a logit link function. All analyses were conducted in the R statistical package version 2.9.2.

*Aporrectodea longa*, typically considered an anecic species, has been recommended classification as an endo-anecic species (Felten and Emmerling 2009) and in exploring differences between surface-living epigeic species and deep-living endogeic species *A. longa* was included in the endogeic category for analyses.

## 5.4 **Results**

Predation by slow worms on earthworms and pulmonates was high, with 65% of slow worms found to have consumed earthworms and 50% found to have consumed either slugs or snails. The proportions of slow worms having consumed various prey species / groups are shown in Table 5.2 along with any significant terms from the GLMs.

Prey Species / Group	Proportion of slow worms having eaten prey	±C.I.	Significant terms in GLM	$\chi^2$	df	P
Earthworms	0.65	0.05	ns			
Epigeic earthworm spp.	0.22	0.04	month mean temperature	26.0 5.6	11 1	0.007 0.018
Endogeic earthworm spp.	0.29	0.04	mean temperature	9.6	1	0.002
<i>Lumbricus rubellus</i>	0.14	0.03	month rainfall	36.1 4.0	11 1	0.001 0.046
<i>L. castaneus</i>	0.13	0.03	month max. temperature	21.8 7.4	11 1	0.026 0.007
<i>L. terrestris</i>	0.16	0.04	ns			
<i>Aporrectodea longa</i>	0.19	0.04	mean temperature	21.4	1	< 0.001
<i>A. caliginosa</i>	0.04	0.02	month	24.0	11	0.013
<i>A. rosea</i>	0.07	0.03	ns			
<i>Allolobophora chlorotica</i>	0.07	0.02	ns			
Pulmonates	0.50	0.05	month months paired †	35.7 14.4	11 5	< 0.001 0.013
<i>Arion</i> spp.	0.30	0.05	month max. temperature sex sex:month paired †	32.0 5.5 11.8 21.2	11 1  10	0.001 0.019 0.003 0.020
<i>Deroceras reticulatum</i>	0.22	0.04	month rainfall	33.0 9.3	11 1	0.001 0.002
<i>Helix aspersa</i>	0.06	0.02	site	10.9	3	0.013

**Table 5.2.** Significant terms from Generalised Linear Models (GLMs) exploring the effects of slow worm body length, weight and sex, site, month, temperature, rainfall and sunshine on predation of prey by slow worms (*Anguis fragilis*) ( $n=400$ ). † indicate results of GLMs considering the effect of the same months combined for 2007 and 2008 instead of analysed separately, in conjunction with all other terms.

#### 5.4.1 Predation on earthworms

For three of the earthworm species, *Lumbricus terrestris*, *Aporrectodea rosea* and *Allolobophora chlorotica*, none of the variables included in the GLM were significant. When earthworms were analysed as a group, no terms were significant either; however, when divided into epigeic and endogeic earthworm categories, mean temperature had a significant effect on predation for both (epigeic:  $\chi^2=5.6$ ,  $df=1$ ,  $P=0.018$ ; endogeic:  $\chi^2=9.6$ ,  $df=1$ ,  $P=0.002$ ), although the effects were different on each, with temperature positively correlated

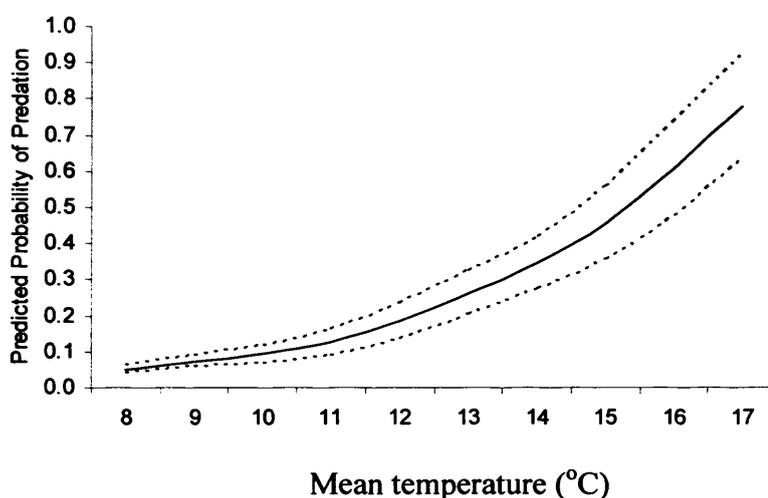
with predation on epigeic species (Fig. 5.1) and negatively correlated with predation on endogeic species (Fig. 5.2). In addition, predation on epigeic species was also significantly effected by month ( $\chi^2=26.0$ ,  $df=11$ ,  $P=0.007$ ), with seasonal patterns of predation on epigeic species shown in Fig. 5.3 and endogeic shown in Fig. 5.4. There was a significant correlation between month and predation on *L. rubellus* ( $\chi^2=36.1$ ,  $df=11$ ,  $P=0.001$ ), *L. castaneus* ( $\chi^2=21.8$ ,  $df=11$ ,  $P=0.026$ ) and *A. caliginosa* ( $\chi^2=24.0$ ,  $df=11$ ,  $P=0.013$ ). Seasonal predation patterns for all seven earthworm species are given in Fig. 5.5.

In addition to month, predation on *L. rubellus* was also significantly negatively correlated with rainfall ( $\chi^2=4.0$ ,  $df=1$ ,  $P=0.046$ , Fig. 5.6). Predation on *L. castaneus* and *A. longa* were both significantly effected by temperature, however the relationship was positive for *L. castaneus* ( $\chi^2=7.4$ ,  $df=1$ ,  $P=0.007$ , Fig. 5.7) and negative for *A. longa* ( $\chi^2=21.4$ ,  $df=1$ ,  $P=0.013$ , Fig. 5.8).

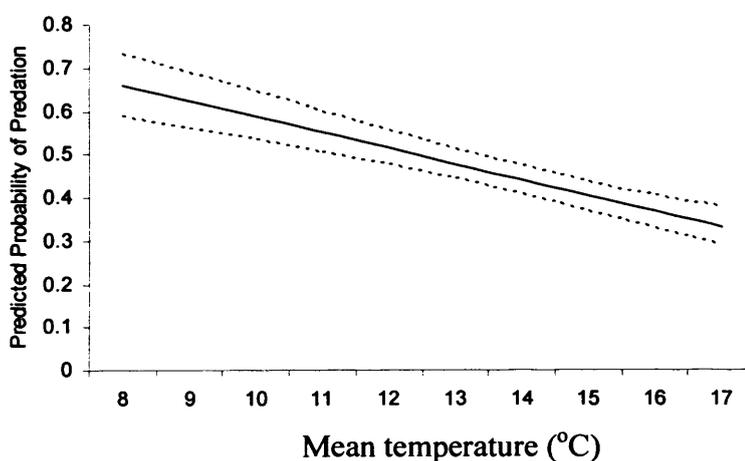
#### 5.4.2 Predation on pulmonate slugs

Predation on *Arion* spp. was correlated with month ( $\chi^2=32.0$ ,  $df=11$ ,  $P=0.001$ ), sex ( $\chi^2=11.8$ ,  $df=2$ ,  $P=0.003$ ) and maximum temperature ( $\chi^2=5.5$ ,  $df=1$ ,  $P=0.019$ , Fig. 5.9). With months combined across both years, there was a significant interaction between month and sex ( $\chi^2=21.2$ ,  $df=10$ ,  $P=0.020$ , Fig. 5.10) with the predicted probability of predation by female slow worms significantly higher than males in April ( $t=-1.94$ ,  $df=382$ ,  $P=0.05$ ), May ( $t=-2.68$ ,  $df=382$ ,  $P=0.01$ ) and September ( $t=-3.02$ ,  $df=382$ ,  $P=0.01$ ). Predation on *Deroceras reticulatum* was also correlated with month ( $\chi^2=33.0$ ,  $df=11$ ,  $P=0.001$ ). Seasonal patterns of predation on *D. reticulatum* and *Helix aspersa* are given in Fig. 5.11. Rainfall had a significant positive effect on predation of *D. reticulatum* (Fig. 5.12). There was a significant

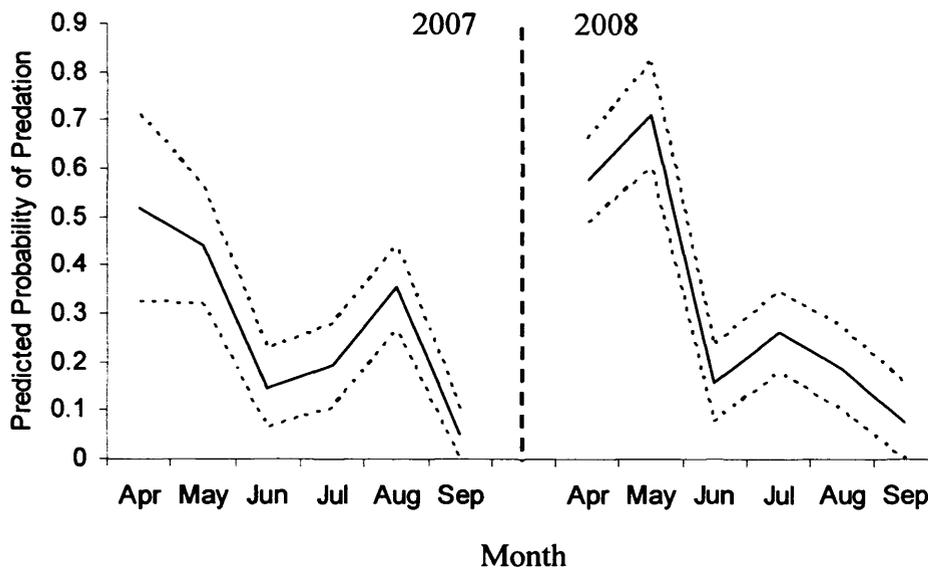
difference in predation on *H. aspersa* between sites ( $\chi^2=10.9$ ,  $df=3$ ,  $P=0.013$ ) with no predation found at Wareham, while 8.2% ( $\pm$ S.E 2.8%) of slow worms at Caerphilly were found to have consumed them, and 6.6% ( $\pm$ S.E 1.8%) of those at Ringwood. While overall there was no significant seasonal effect on predation of earthworms, there was a seasonal effect on predation of pulmonates ( $\chi^2=14.4$ ,  $df=5$ ,  $P=0.013$ , Fig. 5.13).



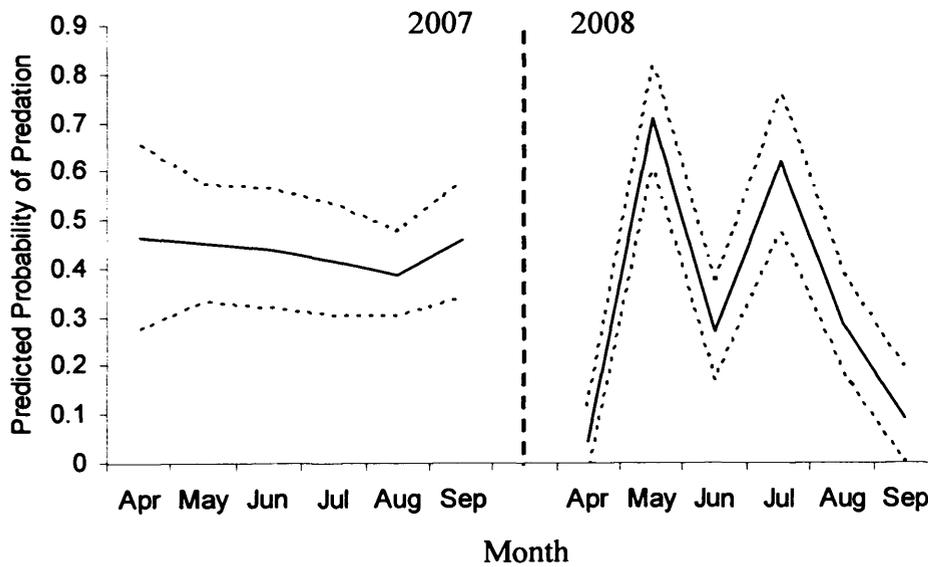
**Figure 5.1.** Effect of mean temperature on the predicted probability of predation (with SE shown by dotted lines) on epigeic earthworms by slow worms (*Anguis fragilis*), as found significant in GLM ( $p=0.018$ ).



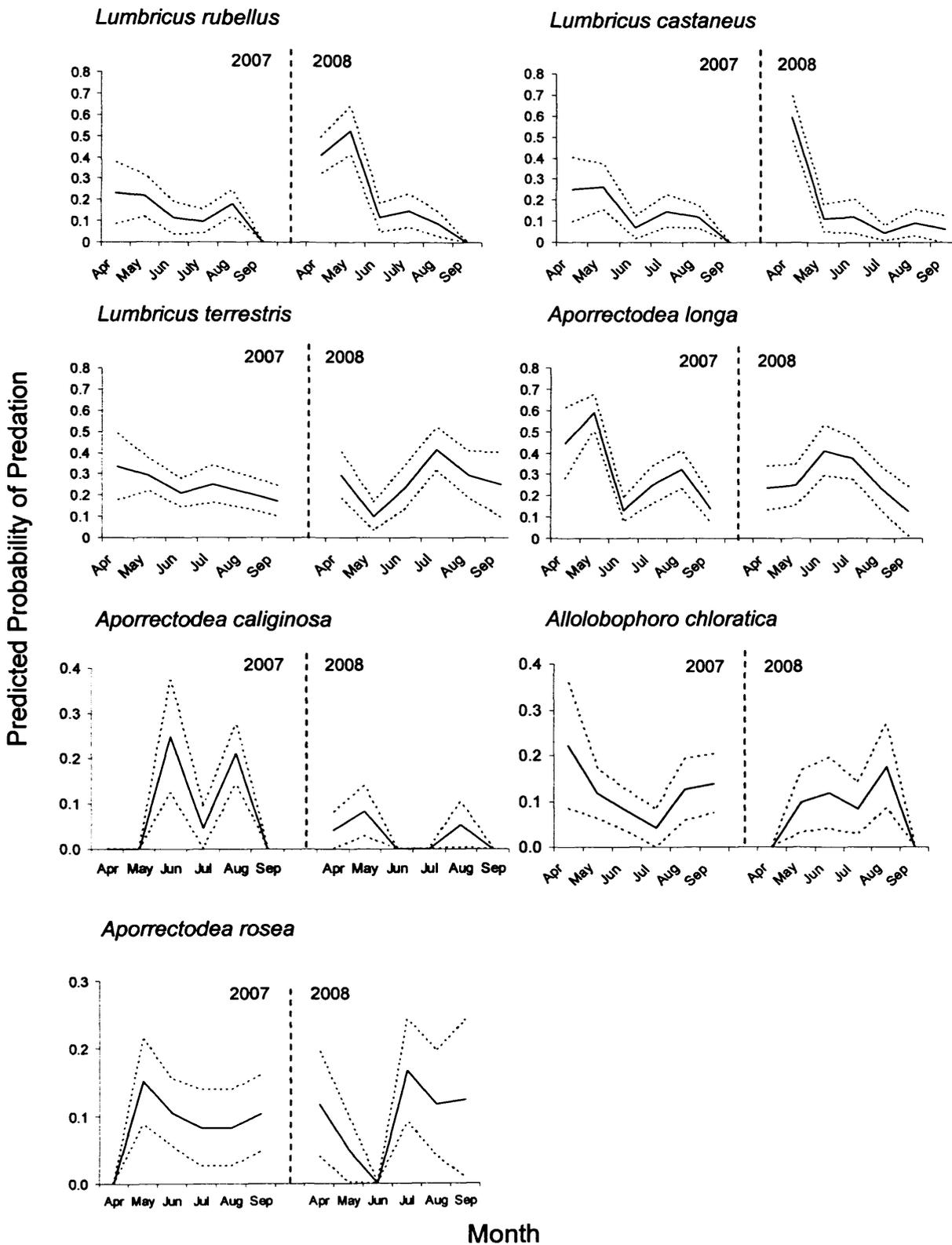
**Figure 5.2.** Effect of mean temperature on the predicted probability of predation (with SE shown by dotted lines) on endogeic earthworms by slow worms (*Anguis fragilis*), as found significant in GLM ( $p=0.002$ ).



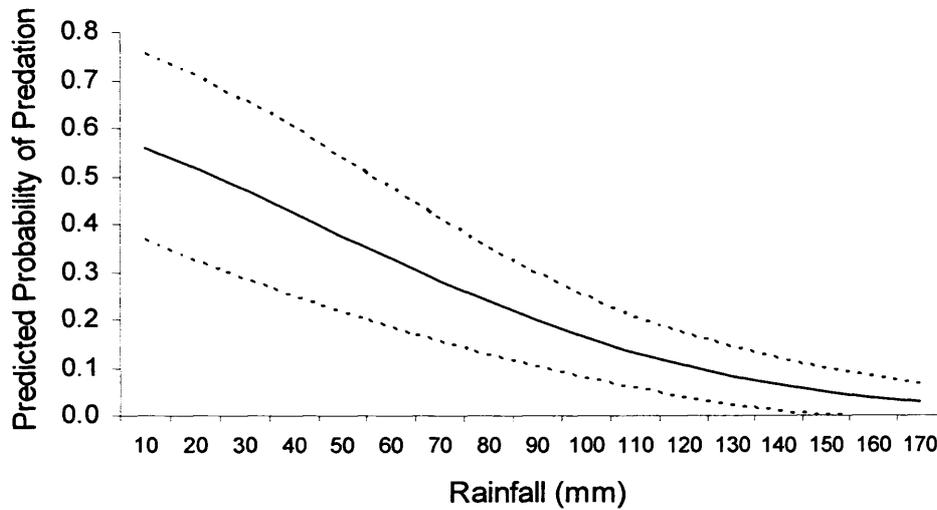
**Figure 5.3.** Temporal changes in predicted probability of predation (with SE shown by dotted lines) on epigeic earthworms by slow worms (*Anguis fragilis*) for 2007 and 2008, as found significant in GLM ( $p=0.007$ ).



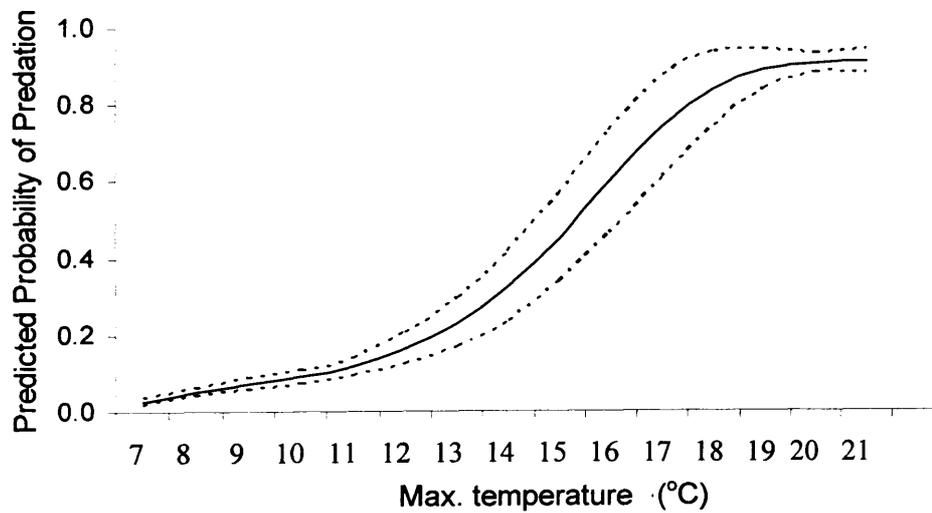
**Figure 5.4.** Temporal changes in predicted probability of predation (with SE shown by dotted lines) on endogeic earthworms by slow worms (*Anguis fragilis*) for 2007 and 2008, as found significant in GLM. No significant difference.



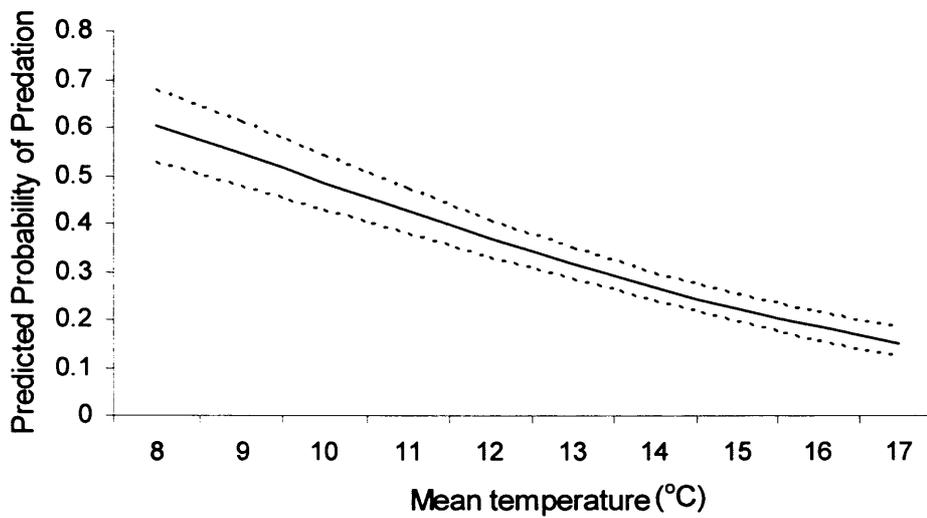
**Figure 5.5.** Predicted probability of predation (with SE shown by dotted lines) on earthworm species by slow worms (*Anguis fragilis*) for 2007 and 2008.



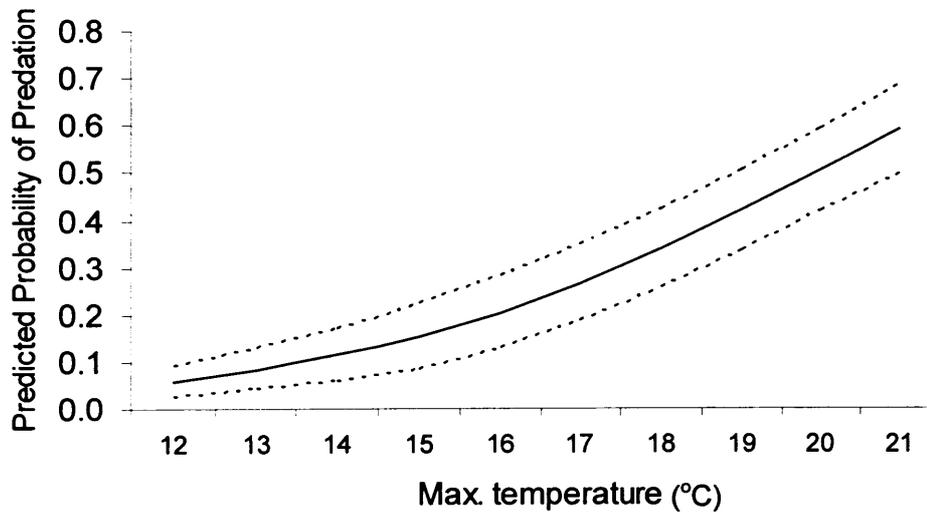
**Figure 5.6.** Effect of rainfall on the predicted probability of predation (with SE bars) on *Lumbricus rubellus* by slow worms (*Anguis fragilis*), as found significant in GLM ( $p=0.046$ ).



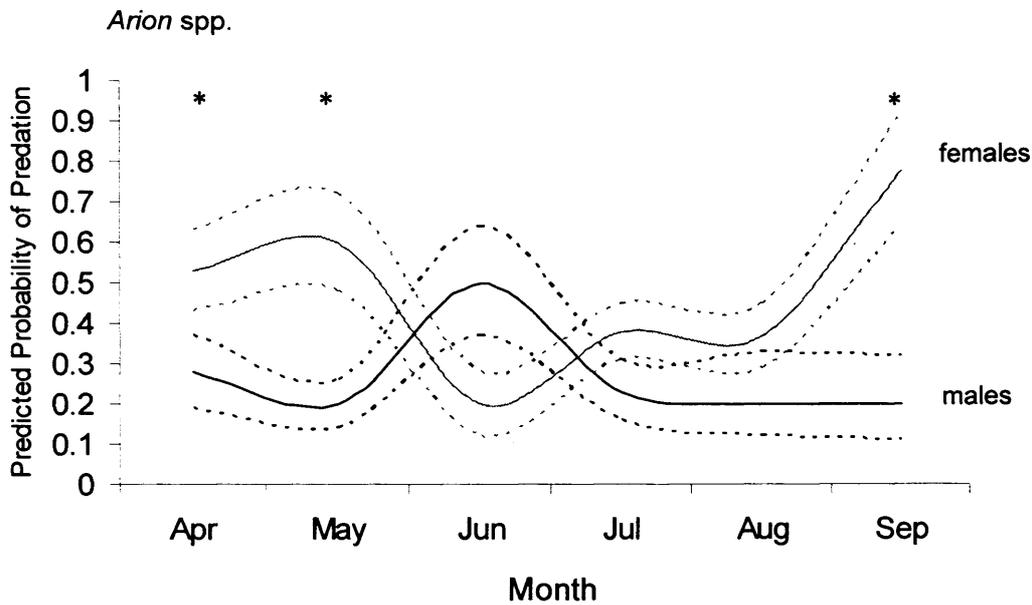
**Figure 5.7.** Effect of mean temperature on the predicted probability of predation (with SE bars) on *Lumbricus castaneus* by slow worms (*Anguis fragilis*), as found significant in GLM ( $p=0.007$ ).



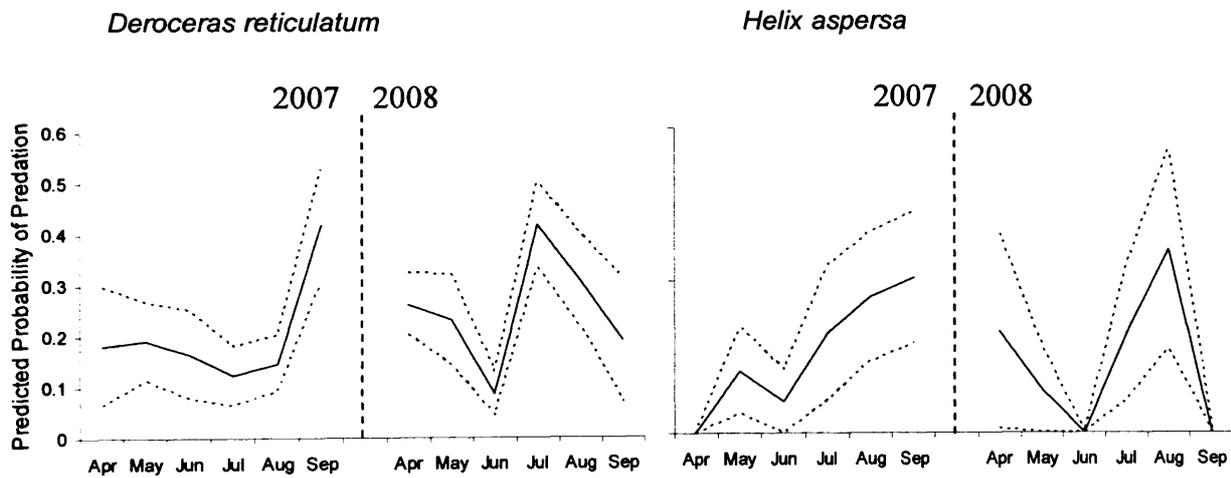
**Figure 5.8.** Effect of mean temperature on the predicted probability of predation (with SE bars) on *Aporectodea longa* by slow worms (*Anguis fragilis*), as found significant in GLM ( $p < 0.001$ ).



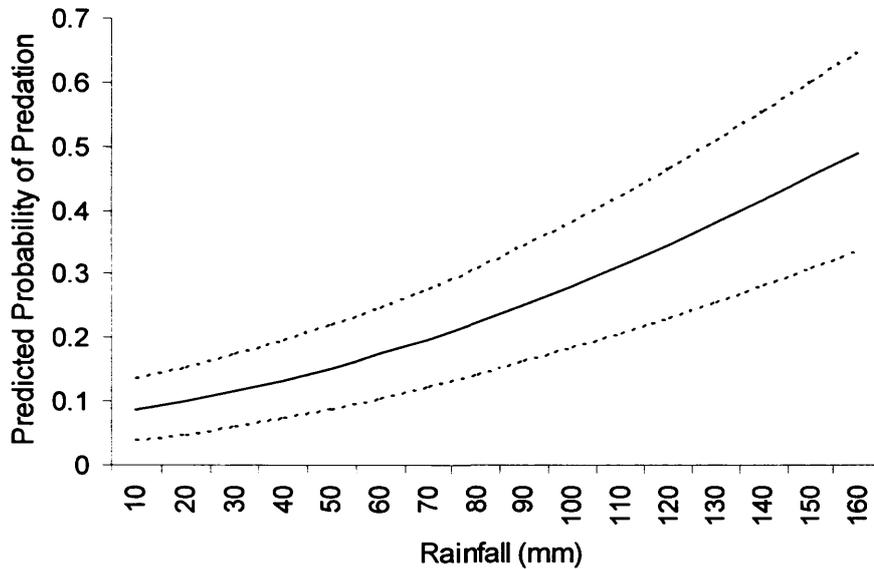
**Figure 5.9.** Effect of maximum temperature on the predicted probability of predation (with SE bars) on *Arion* spp. by slow worms (*Anguis fragilis*), as found significant in GLM ( $p = 0.019$ ).



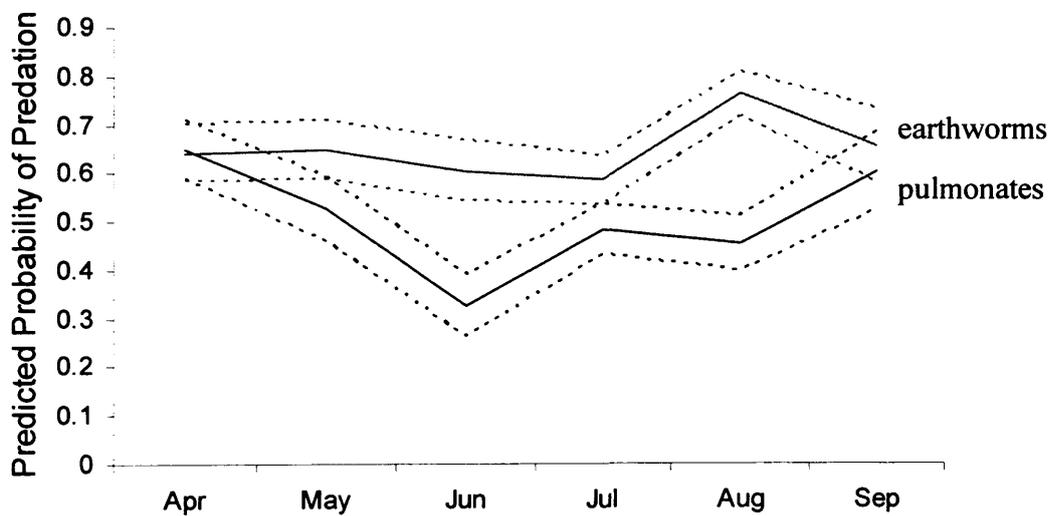
**Figure 5.10.** Temporal changes (for 2007 and 2008 combined) in the predicted probability of predation (with SE shown by dotted lines) by male and female slow worms (*Anguis fragilis*) on *Arion* spp., as found significant in GLM ( $p=0.020$ ).  
\* indicates significant difference between males and females as determined by GLM.



**Figure 5.11.** Temporal changes in predicted probability of predation (with SE shown by dotted lines) by slow worms (*Anguis fragilis*) on *Deroceras reticulatum* ( $p=0.001$ ) and *Helix aspersa* (no significant difference), as found significant in GLM.



**Figure 5.12.** Effect of rainfall on the predicted probability of predation (with SE bars) on *Deroceras reticulatum* by slow worms (*Anguis fragilis*), as found significant in GLM ( $p=0.002$ ).



**Figure 5.13.** Monthly changes (for 2007 and 2008 combined) in the predicted probability of predation (with SE shown by dotted lines) by slow worms (*Anguis fragilis*) on pulmonates ( $p=0.013$ ) and earthworms (no significant difference), as found significant in GLM.

## 5.5 Discussion

Predation by slow worms on earthworms and pulmonates was high (65% and 50% respectively), corroborating the importance of these taxa as prey as found in continental European studies (Luiselli 1992; Pedersen, Jensen and Toft 2009), and suggesting higher rates of predation on them than previously found using morphological rather than molecular diagnostic techniques. This was especially true of earthworms which were found to make up just 33% and 21% of the prey items found in Luiselli's (1992) and Pederson, Jenson and Toft's (2009) studies respectively. Our results may represent a genuine difference in the importance of earthworms in UK slow worm populations, compared with those investigated in Italy and Denmark, or they may be a result of the poor detection success afforded by visual identification when compared with molecular identification. While a molecular approach may be more sensitive, proportions of prey cannot be directly compared to one another as the different primers used may have different amplification efficiencies, and different species may have different susceptibility to digestion. In addition, variation in copy number of mitochondrial genes in cells from different species could affect results (Prokopowich *et al.* 2003), although this is more a problem when comparing very different taxa; it is unlikely that there would be much variation between earthworm species. Differences of prey species in susceptibility to digestion, however, is not unique to molecular detection but also a factor affecting conventional visual identification of prey in regurgitates, faeces or gut dissections.

### 5.5.1 Earthworms

Pederson, Jenson and Toft (2009) found no ontogenetic, seasonal or sex differences in consumption of earthworms by slow worms. Here we were able to explore predation patterns more comprehensively due to larger sample sizes, consideration of numerous abiotic and biotic variables (such as weather and body measurements), and the ability to investigate predation on individual species as well as groups. In investigating ontogenetic patterns of predation, juvenile and adult age classes were used, as used by Pederson, Jenson and Toft (2009), but with the inclusion of a sub-adult category. In addition to this, using snout-vent length as a surrogate for age, a more fine-tuned ontogenetic analysis could be achieved.

No ontogenetic, seasonal or sex effects on predation were detected when earthworms were analysed as a group, but when individual species were considered there were strong seasonal patterns for some. When divided into ecological groups of epigeic and endogeic species, there was a strong effect of month on predation in epigeic species, such as *Lumbricus rubellus* and *L. castaneus*, with predation mostly occurring in the spring. Conversely, predation on deep-living species, such as *Aporrectodea longa* and *A. caliginosa* occurred primarily in the summer, along with predation on *L. terrestris* (an anecic species). This may have been a result of a decrease in the availability of epigeic species, which may have driven slow worms into adopting their nocturnal foraging strategy, which is atypical of most temperate reptiles (see Chapter 4). Without measuring availability of earthworm species it is not possible to make conclusions about prey selection. However, Pedersen, Jenson and Toft (2009) found most prey was taken in accordance with its availability, suggesting slow worms are opportunists, in which case our results would represent seasonal

shifts in earthworm density/availability with predation on them switching between species throughout the year accordingly.

There was a negative correlation between temperature and predation on endogeic species. Endogeic species are likely to remain underground during higher temperatures (Sims and Gerard 1985) to avoid desiccation and therefore be less accessible as temperature increases. For epigeic species, however, predation increased with temperature and decreased with rainfall. Epigeic species are likely to be better adapted to higher temperatures than deep-living species and hence their proportional availability may increase as availability of endogeic species decreases. Additionally, slow worms are more active as temperature increases and are therefore more likely to encounter them. Rainfall, conversely, is likely to attract endogeic species up to the surface, reducing the proportional dominance of surface-living species. Rainfall also brought other alternative prey to the surface, such as slugs (Figs 5.9 and 5.12).

While these results support our hypotheses of there being no ontogenetic or sex differences in relation to predation on earthworms, there are clear strong seasonal effects possibly relating to earthworm surface activity.

### 5.5.2 *Pulmonates*

There was a significant effect of month on predation of pulmonates, with predation higher in the spring and the autumn than in the summer, when slugs numbers are known to be at their highest (e.g. Barker 1991). This seasonal trend was apparent for *Deroceras reticulatum*, *Arion* spp., and *Helix aspersa*, although it was not significant for the latter. Predation on *D. reticulatum* was also positively influenced by rainfall. Meteorological conditions influence

slug activity and population cycles (Rollo 1982; Cook and Ford 1989; Young and Port 1989), with soil moisture and temperature found to increase egg-laying by *D. reticulatum* (Willis *et al.* 2008) and slug density found to be highly correlated with rainfall and temperature (Choi *et al.* 2004). The increase in predation correlated with rainfall, then, is most likely due to increased abundance or activity of *D. reticulatum*. Predation on *Arion* species was not significantly influenced by rainfall but was instead positively correlated with temperature. Temperature is known to affect the density of *Arion* species more than rainfall (Webley 1964; Crawford-Sidebotham 1972), so these results are likely to be a response of predation to increased slug abundance or availability. Furthermore, like with all reptiles, slow worm activity increases with temperature, and this would increase their encounter rates with slugs when temperature was higher.

Predation on *Arion* was found to differ between males and female slow worms, with consumption by females significantly higher early and late in the year (April, May and September). Male and females share territories and are usually found together throughout the year, so it is unlikely that the difference in diet is a result of differential exposure. Instead, it may indicate different nutritional needs of males and females in the spring (during the breeding season) and in the autumn (when offspring are born). If it were simply a result of an increase in overall prey consumption in females then it would be expected to be seen for other prey species too. As this was not the case it may reflect a higher nutritional value of *Arion* species to females and preferential predation upon them, possibly a result of differences in reproductive costs between males and females. Choice experiments have revealed that slow worms preferentially consume *D. reticulatum* over some *Arion* species: they consistently eat *D. reticulatum* in preference to *A. rufus* or *A. distinctus* when offered a

choice (Howes 1986). When offered single species in isolation, *D. reticulatum* was eaten every time (23 out of 23 times), whereas *Arion* spp. was only taken 19% of the time (3 out of 16). In our study, the lower proportion of slow worms found to have preyed upon *D. reticulatum* (22%) compared to *Arion* (30%) may be a result of a lower efficiency of the *D. reticulatum* primers or may reflect low density of this species in comparison to *Arion* species. However, Howes (1986) choice experiment does not state the sex of slow worms that were used, nor when during the season the experiments were conducted, and this may have influenced the findings. Female slow worms may have an innate preference for *Arion* species (specific or all species) in spring and autumn, but at other times prefer *D. reticulatum*.

*Helix aspersa*, the only snail included in the study, was detected in just 6% of slow worms, and none of those from Wareham. As the site at Wareham is open, unlike the more dense vegetation found at Caerphilly and Ringwood, it is probable that *H. aspersa* was either absent from the site or at a lower density.

While these results support our hypothesis of no ontogenetic effects on predation, strong seasonal effects were seen. Our hypothesis of no difference in slow worm predation in relation to sex was also false with regards to *Arion*, for which a preference by females was found.

## 5.6 Conclusions

This study has confirmed the importance of earthworms and pulmonates in the diet of slow worms using a non-invasive molecular approach allowing species-level identification. Overall there are no ontogenetic, seasonal or sex differences in predation on earthworms,

when analysed separately there are distinct seasonal patterns of predation on individual earthworm species, presumably in relation to their availability. This highlights the benefit of DNA techniques in allowing species discrimination and a more thorough analysis. It clearly demonstrated that slow worms were not limited to surface-dwelling earthworm species but could access different ecological groups of earthworms. Temperature and rainfall influence predation on specific species, probably by their influence upon the availability and density (activity-density) of those prey, or on the activity of slow worms themselves. Seasonal effects were also detected in predation on pulmonates, with predation greatly reduced in the summer. Predation on *Arion* was more frequent by female slow worms than males in spring and autumn, possibly the result of differences in reproductive costs leading to differential selection by females. The patterns of predation appear to indicate that predation is predominantly on epigeic earthworms and slugs in the spring, on endogeic earthworms increasingly during the summer, and primarily on slugs in the autumn.

This study confirms that, for earthworms and pulmonates, predation does not change throughout a slow worm's lifetime. As Pederson, Jenson and Toft (2009) suggested, this is most probably because these prey are available in all sizes, in addition to slow worms being capable of taking mouth-sized bites out of larger prey items in these categories.

## 5.7 References

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## **Chapter 6**

**First record of *Neoxysomatium brevicaudatum* in the UK through the non-invasive sampling of *Anguis fragilis*: complementary morphological and molecular detection**

(Submitted to Journal of Helminthology)

**First record of *Neoxysomatium brevicaudatum* in the UK through the  
non-invasive sampling of slow worms (*Anguis fragilis*):  
complementary morphological and molecular detection**

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## 6.1 Abstract

A helminthological investigation of 100 individuals of *Anguis fragilis* was undertaken at 10 locations across Wales. Examination of non-invasively collected faecal samples revealed the presence of *Neoxysomatium brevicaudatum* and another unidentified gastrointestinal nematode. *N. brevicaudatum* was present in 8 out of 12 populations at an overall 38% prevalence, 70.9 mean intensity. Morphological identification was confirmed by sequencing of the 18S ribosomal gene. The use of species-specific nematode primers was found to be an efficient alternative to conventional screening for parasites under the microscope. Although previously unrecorded from slow worms in the UK, it is likely that this parasite is common among slow worm populations.

## 6.2 Introduction

Slow worms (*Anguis fragilis*) are a protected reptile species in the UK, requiring translocation of animals prior to land development. Parasite communities have never before been examined in UK slow worms, yet it may be an important consideration for translocation programmes and the choosing of appropriate receptor sites.

Through the analysis of 100 slow worm faecal samples, collected non-invasively from across ten locations in Wales, two gastroenterological parasitic nematodes were discovered: *Neoxysomatium brevicaudatum* (Zeder 1800) and an unidentified member of the *Rhabdiasidae* family. From dissections of slow worms in Eastern Europe, nine species of parasitic nematode have previously been recorded (Shimalov, Shimalov and Shimalov 2000; Borkovcova and Kopriva 2005; Mihalca *et al.*, 2007), with prevalence of *N. brevicaudatum* found to be 11% (Shimalov, Shimalov and Shimalov 2000) and 43% (Borkovcova and Kopriva 2005), and the number per host ranging between three and ten. Three nematode species belonging to *Rhabdiasidae* were reported in the same studies:

*Paraentomelas dujardini* (Maupas 1916), *Entomelas entomelas* (Dujardin 1945) and *Rhabdias fuscovenosus* (Railliet 1899).

In this study we use non-invasive faecal analysis to investigate prevalence and intensity of parasitic nematodes in slow worms in the UK. Furthermore, we demonstrate the accuracy of a PCR-based approach for detecting *N. brevicaudatum* in faeces.

## 6.3 Materials and Methods

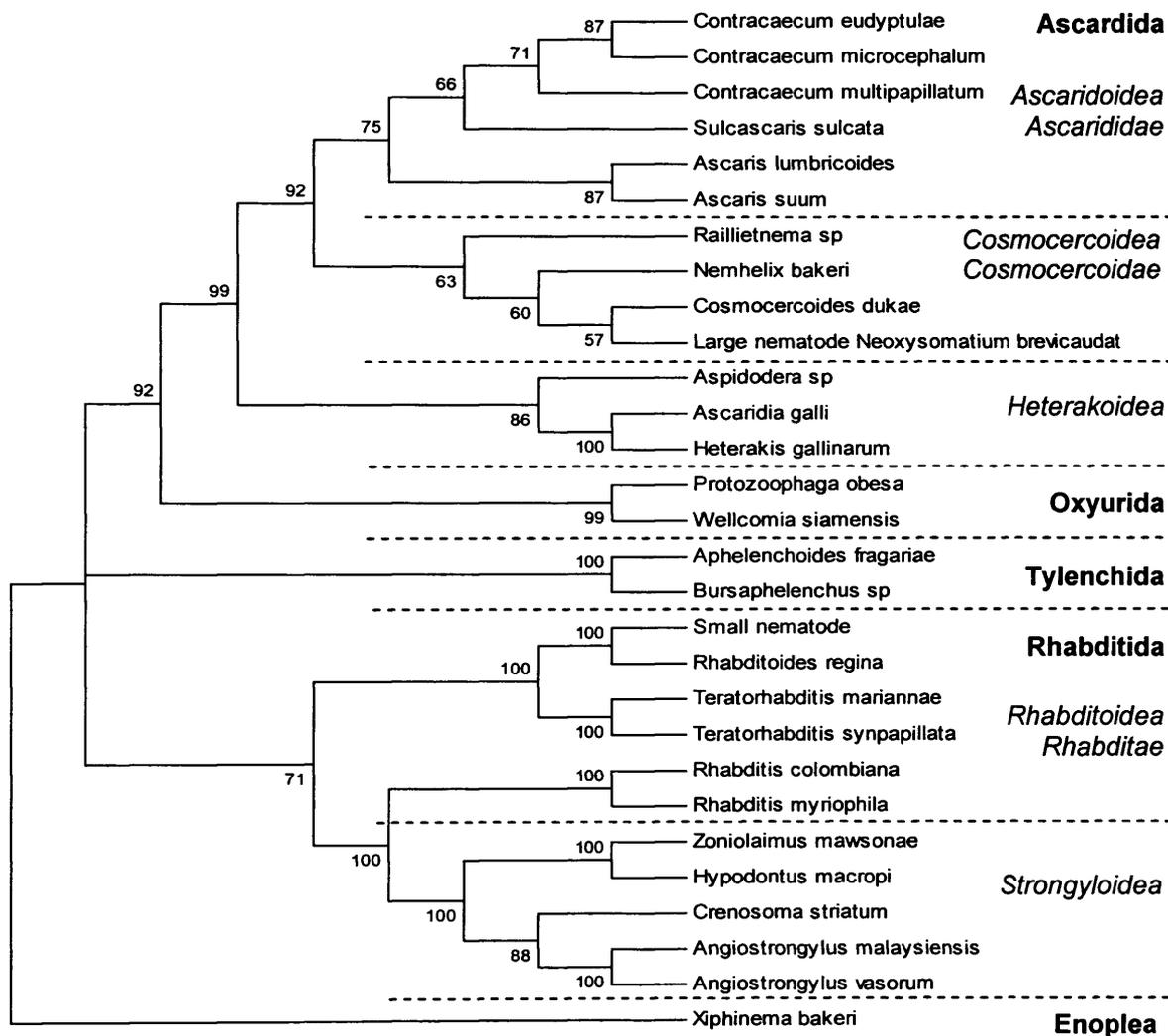
### 6.3.1 Morphological identification

During the course of a long term study on slow worm translocations, we collected faecal samples from 100 specimens of the slow worm (*Anguis fragilis*) between 2006 and 2009. Animals recovered from artificial refugia were photographed, measured and weighed. During this brief handling period, approximately one third of lizards naturally defecated and these samples were stored in sterile vials containing 90% ethanol. Once returned to the laboratory, each sample was poured into small glass Petri dishes and examined on the stage of a zoom (up to x30 magnification) dissecting microscope. Two nematode species were recovered and counted. They were cleared in beechwood creosote and examined under a high-power Olympus microscope at the Natural History Museum, London. The larger of the two was identified as *Neoxysomatium brevicaudatum* (Zeder, 1800).

### 6.3.2 Molecular identification

Nematode DNA was extracted using the DNeasy Tissue Kit (Qiagen) following the Animal Tissues protocol. DNA was amplified in PCR with 18S rDNA primers r1f and 1500R (Tkach *et al.*, 2006) using the following conditions: 1X buffer, 2 mM MgCl<sub>2</sub>, 0.1 mM dNTPs (Invitrogen), 0.5 µM of each primers, 0.45 U *Taq* polymerase (Invitrogen) and 5 ng/µL DNA in a total volume of 25 µL, with an initial denaturation at 94 °C for 3 min, 45 cycles of 94 °C for 20 s, 47 °C for 1 min and 72 °C for 1 min, and a final

extension at 72 °C for 5 min. Sequencing was performed on an ABI 3100 automated capillary DNA analyser (ABI Prism model 3100, Beaconsfield, UK). Comparison of the 588 bp sequence (Accession No. 1360153) of the larger nematode with those on Genbank returned a closest match of 99% similarity to *Cosmocercoides dukae*. Construction of a neighbour-joining tree in Mega 3.1 using sequences from 39 nematode species downloaded from Genbank (representative of 5 orders and 11 families) confirmed its place in the Cosmoceroidea family. The smaller nematode grouped within the Rhabditidae family, matching closest at 96% with *Rhabditoides regina* (Fig. 1).



**Figure 1.** Phylogenetic tree showing the relationship of the two nematodes found in UK slow worm faeces with other nematode species, the larger nematode identified as *Neoxysomatium brevicaudatum* and the smaller nematode unidentified. The tree was constructed based on 588 bp of the 18S rDNA sequence by using the neighbour-joining method. The bootstrap analysis was performed with 1000 repetitions.

### 6.3.3 Molecular screening

DNA was extracted from an additional ten slow worm faecal samples (collected from Verwood, Dorset ( $n=8$ ) and Caerphilly, Vale of Glamorgan ( $n=2$ )) using the QIAamp® DNA Stool Mini Kit (Qiagen) in accordance with the manufacturer's instructions. For the initial step of the protocol, the faecal sample was vortexed and lysed in buffer for 1 min, with the remainder of the extraction process carried out on the supernatant. This brief lysis is sufficient to extract nematode DNA without damaging the specimen, allowing for post-extraction morphological identification under a microscope. DNA extraction success was established by screening with the universal primers LCO1490 (Folmer *et al.*, 1994) and C1-N-1777 (described in Section 2.3.3.2) which amplify a 287 base pair fragment of the Cytochrome Oxidase I (COI) mitochondrial gene. PCR conditions were as previously described. Amplification success of DNA was confirmed by gel electrophoresis. With all extractions testing positive for DNA, *N. brevicaudatum*-specific primers COI-J-1764 and COI-N-1938 (Brown *et al.*, unpublished), which amplify a 174 base pair fragment of the COI mtDNA gene, were used to amplify DNA from any *N. brevicaudatum* nematodes extracted from the faecal samples. PCR conditions were as previously described, but with an initial denaturation at 94°C for 5 min, 40 cycles of 94°C for 30 s, 66°C for 45 s and 72°C for 45 s, and a final extension at 72°C for 10 min. All PCRs were performed on a Peltier Thermal Cycler (Bio-Rad Laboratories, CA, USA).

## 6.4 Results

Screening of faecal samples from Welsh captured slow worms revealed two gastrointestinal nematode species, but no other macroparasites. The larger nematodes were identified as *Neoxysomatium brevicaudatum* (Zeder, 1800) (prevalence 38%, mean intensity 70.9, range 1-686; Table 1). There were insufficient morphological characters to

explicitly identify the smaller nematode, however molecular identification placed it in the Rhabditae family (prevalence 83%, mean intensity 102.8, range 1-2000).

Site	<i>Neoxysomatium brevicaudatum</i>			Rhabditae nematode		
	Prevalence	Mean intensity	Range	Prevalence	Mean intensity	Range
1. Aberamen	1/4 (25%)	1	1	3/4 (75%)	25.3	18-38
2. Llandysul	1/4 (25%)	1	1	3/4 (75%)	22.3	19-28
3. Llysdinam	2/2 (100%)	1	1	1/2 (50%)	5	5
4. Machen	0/2 (0%)			2/2 (100%)	23	22-24
5. Pentwyn	0/3 (0%)			3/3 (100%)	9	3-20
6. Monmouth	0/3 (0%)			2/3 (66%)	10.5	1-20
7. Pencoed	3/9 (33%)	1	1	8/9 (89%)	47.8	2-253
8. Pontypool	1/12 (8.3%)	29	29	9/12 (75%)	12.1	1-41
9. St Athan	8/11 (72.7%)	53.5	1-173	11/11 (100%)	304.8	4-1285
10. St Bride's Major	22/50 (44%)	101	1-686	41/50 (82%)	108.4	2-2000
<b>Total</b>	<b>38/100 (38%)</b>	<b>70.9</b>	<b>1-686</b>	<b>83/100 (83%)</b>	<b>102.8</b>	<b>1-2000</b>

**Table 1.** *Neoxysomatium brevicaudatum* and unidentified gastrointestinal nematode infections prevalence, mean intensity and range) from *Anguis fragilis* (n = 100) in Wales, U.K.

From a further ten slow worm faecal samples screened with *N. brevicaudatum*-specific COI primers, four tested positive. Subsequent examination of the same faecal samples by microscopy recorded these four samples as having at least one *N. brevicaudatum* present in each (1-2 adults), in addition to the smaller nematodes (range 66-861) Of the six faecal samples that were negative by molecular screening, none were found to contain any *N. brevicaudatum*, but four did contain the smaller nematodes (range 28-31).

## 6.5 Discussion

For the first time *Neoxysomatium brevicaudatum* was recorded from UK slow worms. This parasite infects the intestine of amphibians (e.g. *Bombina*, *Bufo*, *Hyla*, *Rana*, *Triturus*) and occasionally reptiles (e.g. *Anguis*, *Natrix*) (see Saglam and Arikan, 2006; Karadeniz *et al.*, 2005; Kirin, 2002; Shimalov and Shimalov, 2000; Shimalov *et al.*, 2000). It has a direct life-cycle, the eggs hatching outside the host and the first stage larvae developing and moulting twice to the infective third stage. It is probable that the final host becomes infected orally, the larvae often being found in the tissues (Saeed *et al.*, 2007; Vashetko *et al.*, 1999). Males range from 3-4 mm in length with the females slightly larger at 4-5 mm. The nematode is ovoviviparous with eggs measuring 0.09 mm x 0.05 mm (Ryzhikov *et al.*, 1980). It is a common pathogen of herptofauna throughout Europe (Saglam and Arikan, 2006; Borkovcová and Kopřiva, 2005; Kirin and Buchvaov, 2002; Sharpilo, 1974; Yamaguti, 1961) but has not apparently been previously recorded from slow worms in the UK. This is yet another example of the current lack of knowledge of the helminth parasites of British vertebrates.

Quantitatively, we would expect much higher parasitic loads from dissected animals, but, non-invasive sampling has provided estimates of parasite load. In the faecal samples screened with *N. brevicaudatum*-specific COI primers, prevalence was determined to be 40%, which was corroborated by microscopy. This indicates that molecular screening is as efficient as conventional methods and offers an alternative to the time-consuming and laborious process of hand-sorting, and one which does not necessitate skilled nematode taxonomists. However, an essential prerequisite of molecular screening was the original morphological identification of the samples, as identification through sequencing alone would only have identified *N. brevicaudatum* to family level. Also, the molecular approach used could only provide information on

prevalence, although other studies have shown real-time PCR as a suitable method for ascertaining intensity (e.g., Nielsen et al., 2008).

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## **Chapter 7**

**Molecular detection of sex and age-biased  
nematode prevalence in slow worms (*Anguis fragilis*)**

**(To be submitted to Molecular Ecology)**

**Molecular detection of sex and age-biased nematode prevalence in slow  
worms (*Anguis fragilis*)**

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Running title: Nematode prevalence in slow worms

Keywords: acquired immunity, age-prevalence, immunosuppression, parasitism, reptiles

## 7.1 Abstract

In vertebrates, the prevalence and intensity of parasitic infections is often higher in males than in females. This bias is frequently explained as representing either differences between host sex in exposure or susceptibility to parasites. Such differences in exposure may be due to sex-specific behaviour of the host, including differential habitat use or differences in diet. Differences in susceptibility are often regarded as a negative effect of sex-steroid hormones (particularly testosterone) on the immune system.

In this study a non-invasive molecular approach, analysis of faeces with species-specific PCR primers, was used to investigate whether there is a sex-bias in prevalence of the gastroenterological parasitic nematode *Neoxysomatium brevicaudatum* in slow worms (*Anguis fragilis*) in the UK, a protected reptile species. Faeces were collected each month (April-September) from three sites over two years. Prevalence was modeled by GLM to investigate the effects of slow worm length, weight, age and sex along with environmental variables such as temperature, rainfall and sunshine.

There was sex-bias with a significantly lower likelihood of prevalence in males than females in August 2007 and July 2008. With years pooled together to increase sample size, a significantly higher male sex-bias in prevalence was found for April ( $p=0.05$ ). As this is during the breeding season for slow worms, it may well represent testosterone-induced immunosuppression. A second order interaction between slow worm length and weight was found to be significant, with a positive association between prevalence and body condition in young slow worms (below 150mm) and a negative association in older slow worms (above 150mm). The convex pattern of nematode prevalence with age suggests an increase with age-related exposure and a decrease with age-related acquired immunity.

## 7.2 Introduction

In vertebrates, the prevalence (percent of hosts infected) and intensity (number of parasites per host) of parasitic infections is frequently found to be higher in males than in females (e.g., Poulin, 1996a; Zuk & McKean, 1996; Schalk & Forbes, 1997; Klein, 2004; Robinson *et al.*, 2008). Commonly, this bias is explained as a function of either differential susceptibility (e.g., Zuk, 1990; Poulin, 1996a; Klein, 2000; Moore & Wilson, 2002) or exposure (e.g., Drobney *et al.* 1983; Tinsley, 1989; Reimchen & Nosil, 2001; Krasnov *et al.* 2005) between host sex.

Differences in susceptibility are often attributed to the well documented negative association between sex-steroid hormones (particularly testosterone) and the immune system (e.g., Folstad *et al.* 1989; Folstad & Karter, 1992; Wedekind & Jacobson, 1998). The production of male secondary sexual traits is governed by testosterone which is simultaneously immunity suppressive. This testosterone-induced immunosuppression (Zuk & McKean, 1996) supports Zahavi's handicap hypothesis (Zahavi, 1975) and Folstad & Karter's (1992) immunocompetence handicap hypothesis (Kurtz & Sauer, 1999) in that the ability to display secondary sexual traits is costly (in terms of increased parasite risk) and therefore an honest signal of male quality, as only the fittest males are able to display with the burden of this handicap. Host androgens not only have indirect effects on parasite abundance via the immune system, but they can also directly affect growth and development of the parasite itself (e.g., Drutz *et al.* 1981; Harder *et al.* 1992; reviewed in Lawrence 1991 and Beckage 1993).

Differences in exposure to parasites may be due to sex-specific behaviour of the host, including differential use of habitat between sexes (e.g. Tinsley, 1989), aggression between

males for mating opportunities (e.g., Mills *et al.*, 1999), aggregation of one sex (Zuk & McKean, 1996), or differences in diet (e.g., Thomas, 1965; Kennedy, 1968; Borgstrom, 1970; Drobney *et al.* 1983; Poole, Chadee & Dick, 1983 ). Furthermore, behavioural differences in one sex which result in stress (e.g., territory defense, intraspecific fighting, energy-intensive courtship displays) may have significant negative effects on the immune system (Stein & Schleifer, 1985), increasing susceptibility, if not exposure, to parasitic infection. Experiments by Noble (1961, 1962, 1966) demonstrated that stress caused by temperature, light intensity, annoyance, crowding and hunger all resulted in increased parasite loads.

Alternatively, exposure is not limited to behaviour and can also be a result of sexual dimorphism. As males are often larger than females they may simply offer a larger contact area for parasites (e.g., Kuris *et al.*, 1980; Hamann *et al.*, 2006) or may ingest greater amounts of infected prey (intermediate hosts) (Poulin, 1996b).

There are plenty of confounding factors to consider, however. For example, if a host accumulates parasites with time and one sex has a higher mortality than the other (e.g., Paling, 1965; Halvorsen & Andersen, 1984) then the high parasite levels of the longer-surviving sex may be misinterpreted as a sex-bias, should age not be accounted for. Season or year, too, may affect susceptibility to parasites or behaviour, influencing exposure (e.g., Schall & Marghoob 1995), in particular changes in activity or diet during the breeding season (Drobney *et al.* 1983). Age and season, then, may need to be accounted for in order to draw meaningful conclusions, but this is seldom the case.

In this study we used a non-invasive molecular approach to investigate whether there is a male sex-bias in prevalence of a gastroenterological parasitic nematode, *Neoxysomatium*

*brevicaudatum*, in slow worms (*Anguis fragilis*) in the UK, a protected reptile species, by analysing faecal samples collected each month (between April and September) over two years from different habitats. Where possible, host age, length and weight were recorded, along with regional mean monthly temperatures and rainfall provided by the Met Office. This allowed for a comprehensive analysis of the parasite-host patterns in this animal. Dissections of Eastern European slow worms have revealed eleven species of parasitic helminths (Shimalov *et al.* 2000; Borkovcova and Kopriva 2005), with prevalence of *N. brevicaudatum* detected as 11% (Shimalov *et al.* 2000) and 43% (Borkovcova and Kopriva 2005), but these findings are constrained by the small sample sizes involved: nineteen and seven respectively. This nematode is not exclusively found in slow worms and has been reported in frogs and toads (Düşen and Öz 2006; Saglam and Arikan 2006). The same is true of all nematode species detected in slow worms (Shimalov *et al.* 2000; Borkovcova and Kopriva 2005). Whether *N. brevicaudatum* is a generalist parasite of both amphibians and reptiles, or an unintentional commensal visitor of slow worms, was also a consideration of this study.

While nematodes are the most abundant animals on earth (Coghlan 2005), they are generally considered to be one of the most difficult to identify to species level. Their identification is traditionally accomplished through microscopy and careful identification of morphologically defining characteristics. However, identification is difficult and requires skilled nematode taxonomists and due to a lack of informative features even with expertise many cannot be identified even to family level (McKeand, 1998). Eggs and early developmental stages present even more of a challenge, with egg identification requiring as many as twenty different parameter measurements (Georgi & McCulloch, 1989; Sommer

1996). Standard procedure is the laborious and time-consuming *in vitro* culturing (coproculture) of eggs through to infective third stage (L3) larvae which are, to varying degrees, morphologically distinguishable by microscopy (Keith, 1953; Burger and Stoye, 1968). Even where nematodes can be identified, quantifying parasitic infection under the microscope is a slow, laborious, tedious procedure that requires a high intensity of infection for good sensitivity. The advent of molecular techniques has overcome many of these constraints and has allowed for quick, detailed analyses by non-nematode taxonomists. Serological techniques (e.g. Enzyme-linked immunosorbent assay (ELISA)) for the detection of host humoral response to parasites have proved to be simple, fast and automatable but suffer from poor specificity and cannot distinguish between antibodies produced in active or latent infections (e.g., De Savigny *et al.* 1979; Grieve *et al.* 1981; van Knapen *et al.* 1981; Kloosterman *et al.* 1993; Lalitha *et al.* 2002). The earliest DNA based approaches were DNA-DNA hybridization assays designed for detecting parasites or their eggs in blood or faecal samples, with a sensitivity to detect 50-100 eggs (Coghlan and Wolfe, 2002; Flisser, *et al.* 1988), but these have been superseded by PCR methods such as RFLP (restriction fragment length polymorphisms) (e.g., Newton *et al.* 1988; Jacobs, *et al.* 1997; Gasser, *et al.* 1999; Nuchprayoon, *et al.* 2006) or the more direct use of parasite species-specific PCR primers (e.g., Newton *et al.* 1998; Romstad *et al.* 1997; Hung *et al.* 1999; Zarlenga *et al.* 1998; Schnieder *et al.* 1999; Verweij *et al.* 2000; Verweij *et al.* 2001; Zarlenga *et al.* 2001; de Grutjter *et al.* 2005; Harmon *et al.* 2007). A constraint of faecal analysis (whether molecular or traditional) is that it detects only parasites that have been shed and will underestimate prevalence unless all infected animals have shed parasites. However, it can

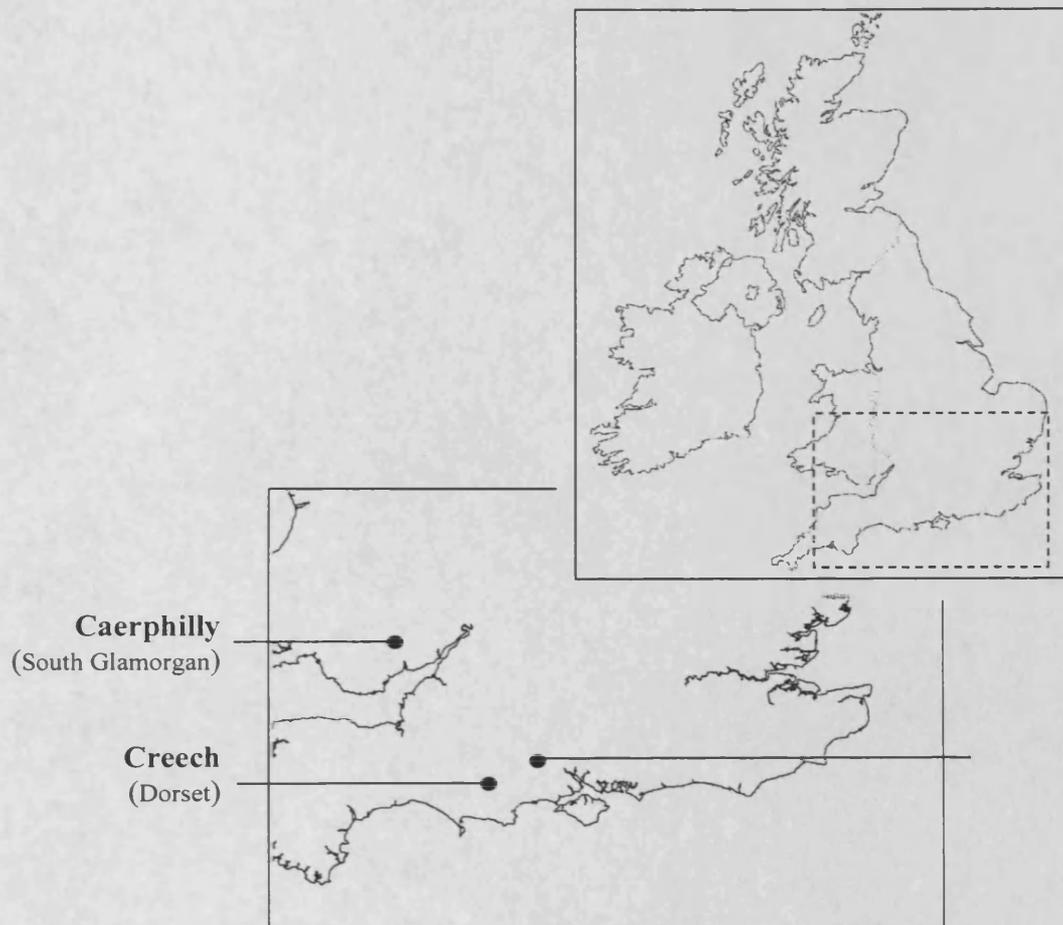
often provide a good estimate of prevalence and is preferable to the invasive alternative of dissection when dealing with vertebrates, particularly ones of conservation concern.

Our approach was to use species-specific PCR primers to detect nematodes in slow worm faeces. We tested the hypotheses that prevalence of *N. brevicaudatum* is sex biased and that it has a negative impact on host body condition.

### 7.3 Method

#### 7.3.1 Study sites and faecal collection

A total of 270 faecal samples were collected from slow worms during monthly visits to three sites (Caerphilly, Ringwood and Creech) with different habitat characteristics between April–September in both 2007 and 2008 (Fig. 7.1). The Caerphilly site is an approximately five hectare area of marshy grassland, comprising purple moor grass (*Molinia caerulea*), tufted hair grass (*Deschampsia cespitosa*), gorse (*Ulex* spp.), bramble (*Rubus fruticosus*) and various fern species (*Dryopteris* spp.) surrounded by areas of species-poor acid grassland. The Ringwood site consists of just under a hectare of unimproved grassland adjacent to Ericaceous heathland and coniferous woodland. Creech is an area of Ericaceous heathland comprising common heather (*Calluna vulgaris*), bell heather (*Erica cinerea*) and gorse (*Ulex* spp.).



**Figure 7.1.** Locations of sampling sites where slow worm faecal samples were collected.

Faecal samples were collected into 2 mm microcentrifuge tubes by gentle palpation of the animals. Effort was taken to collect faeces from males and females at each sampling time, both adults and subadults and, where possible, juveniles. Snout-vent length and total weight were measured, and the presence / absence of a complete tail recorded as an indication of a predatory attack.

### 7.3.2 *Nematode DNA*

DNA was extracted from whole nematode specimens found in slow worm faeces using the DNeasy® DNA Tissue Kit (Qiagen) in accordance with the manufacturer's instructions. PCR primers LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer *et al.* 1994) were used to amplify a c700 bp region of the cytochrome oxidase I (COI) mitochondrial gene, using the following conditions: 1X buffer, 2 mM MgCl<sub>2</sub>, 0.1 mM dNTPs (Invitrogen), 0.5 µM of each primer, 0.45 U *Taq* polymerase (Invitrogen) and 5ng/µL of DNA with an initial denaturation at 94°C for 3 min, 45 cycles of 94 °C for 30 s, 46 °C for 1 min and 72 °C for 1 min, and a final extension at 72 °C for 5 min. Nematode DNA was additionally amplified with 18S rDNA forward primer rift (5'-GCGGCTTAATTTGACTCAACACGG-3') and reverse 1500R (5'-GCTATCCTGAGGGAACTTCG-3') (Tkach, Kuzmin & Pulis 2006) using the same PCR conditions as above. PCRs were run on a Peltier Thermal Cycler (Bio-Rad Laboratories, CA, USA) and PCR products were sequenced with an ABI 3100 automated capillary DNA analyzer (ABI Prism model 3100, Beaconsfield, UK). Construction of a neighbour-joining tree with COI and 18S sequences allowed molecular confirmation of the nematode species.

### 7.3.3 *PCR primers design and testing*

Homologous COI sequences to *N. brevicaudatum* from a range of nematode taxa (see Table 7.1) were acquired from the Genbank database and aligned in BioEdit 7.0.4.1 (Hall, 1999) for the design of *N. brevicaudatum*-specific primers. NetPrimer (Biosoft International) was used to test primer sequences for potential primer-dimer and hairpins which would reduce

<b>Nematode species</b>	<b>Order &gt; Superfamily</b>	<b>5' nucleotide position: 1764</b>	<b>5' nucleotide position: 1938</b>
<i>Neoxysomatium brevicaudatum</i>	Ascaridida > Cosmocercoidea	5'-TCTTAGATTTTGACTTTTGCCCTACAG-3'	3'-CTAATGTAACACGACCACAATCAAGA-5'
<i>Heterakis isolonche</i> (FJ009627)	> Heterakoidea	.A...T....GT.G....A..TT	.T.....T.....A.....
<i>Anisakis simplex</i> (AY994157)	> Ascaridoidea	.T.A..T....GT.A.....T.	.T.....C.....G.....
<i>Toxocara cati</i> (AM411622)	> Ascaridoidea	.T.G..T....GT.G.....G.	.A.....C.....
<i>Anisakis pegreffii</i> (FJ907317)	> Ascaridoidea	C....C....GT.G.....T.	.AG.....C.....
<i>Rostellascaris</i> sp. (EU741046)	> Ascaridoidea	.T.A..T....GT.A.....	.A.....T.....
<i>Ascaris suum</i> (X54253)	> Ascaridoidea	.T.A..T....GT.G.....T.	.A..C.....C....T..N
<i>Necator americanus</i> (AF303157)	Rhabditida > Ancylostomatoidea	.T.A..T....GT.A..A....T.	.A.....A.T.....
<i>Parelaphostrongylus odocoilei</i> (EF173699)	> Metastrongyloidea	CT.A.....T.A..A....TT	.....A.T....T
<i>Steinernema arenarium</i> (AY943979)	> Panagrolaimoidea	..A..T..C...T.A..A....T.	.A.....G...T.....C
<i>Steinernema cubanum</i> (AY943983)	> Panagrolaimoidea	.T.A.....GT.A..A....T.	.AG.A.....G...T.....
<i>Steinernema carpocapsae</i> (AY943981)	> Panagrolaimoidea	.T.A..T....T.GC.A..A..T.	.AG.A.....T..TT...G..T
<i>Strongylus equinus</i> (U57038)	> Strongyloidea	..A..T...G..T.G..A..C..T.	.A..C.....T..A..TCA..
<i>Diplogastrellus metamasius</i> (EU419761)	> Rhabditoidea	.T.A.....T.A..A..A..T.	.A.....CA.T....T
<i>Oigolaimella</i> sp. (AB478634)	> Rhabditoidea	.T.A.....T.G..A..A..TT	.A.....A.T....T
<i>Rhabdias okuensis</i> (FM179479)	> Rhabditoidea	.A.A..T....T.A..A.....	.A.....T....C..T
<i>Rhabdias mariauxi</i> (FN395319)	> Rhabditoidea	.A.A..T....T.AC.....	.A.....TT....C..T
<i>Mecistocirrus digitatus</i> (AB245051)	> Trichostrongyloidea	.T.G..T....T.G..A....G.	.A.....A..TCT..
<i>Haemonchus contortus</i> (EU346694)	> Trichostrongyloidea	.T.A.....T.G..A....T.	.AG.A.....C...A..TCT..T
<i>Nippostrongylus brasiliensis</i> (U57035)	> Trichostrongyloidea	.T.A..T....T.A..A....T.	.A.....A.C...A..TCA..T
<i>Heligmosomum mixtum</i> (DQ408635)	> Trichostrongyloidea	.T.G..T....GT.A..A..A..T.	.....A..TCA..T
<i>Bursaphelenchus poligraphi</i> (AY508059)	Tylenchida > Aphelenchoididae	.T.A....C...T.A..A....TT	.A..C.....C...TCA.A.
<i>Bursaphelenchus seani</i> (AY508061)	> Aphelenchoididae	.T.A.....A...TTT	.AG.A.....C...TCA..T

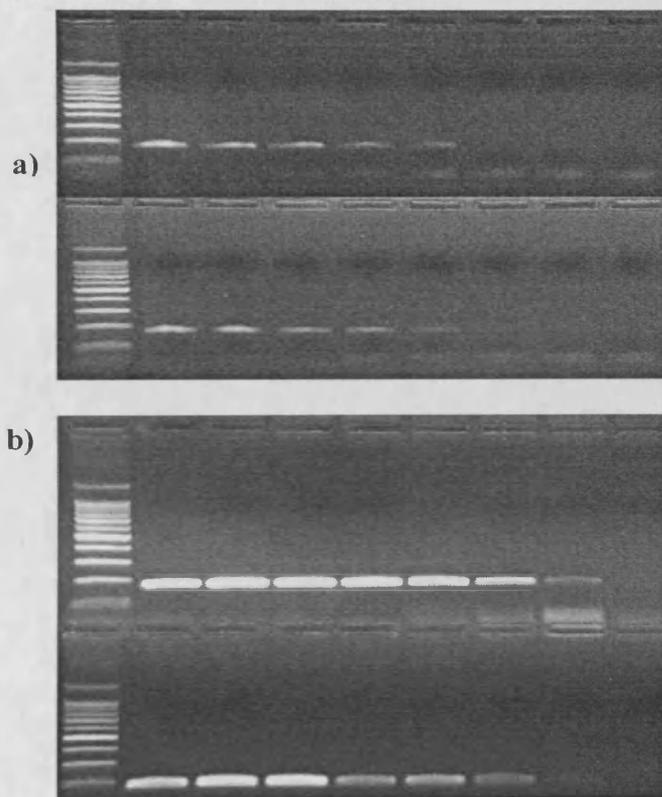
**Table 7.1.** Cytochrome Oxidase I forward and reverse primer sites for *Neoxysomatium brevicaudatum* aligned with other nematode taxa.

primer efficiency. Of the primers designed, COI-J-1764 (5'-TCTTAGATTTTACTTTTGCCTACAG-3') and COI-N-1938 (5'-AGAACTAACACCAGCACAATGTAATC-3'), which amplify a 174 bp fragment, were species-specific when tested for cross-amplification against a range of species (Table 7.2), such as the slow worm itself, potential prey (annelids, gastropods and arthropods), and nematode species. PCR conditions were: 1X buffer, 2 mM MgCl<sub>2</sub>, 0.1 mM dNTPs (Invitrogen), 0.5 μM of each primer, 0.45 U *Taq* polymerase (Invitrogen) and 5ng/μL of DNA with an initial denaturation at 94°C for 5 min, 40 cycles of 94°C for 30 s, 66°C for 45 s and 72°C for 45 s, and a final extension at 72°C for 10 min.

<b>Species</b>	<b>Phylum</b>	<b>Order</b>	<b>Family</b>
<i>Anguis fragilis</i>	Chordata	Sauria	Anguidae
<i>Coronella austriaca</i>	Chordata	Squamata	Colubridae
<i>Lumbricus rubellus</i>	Annelida	Haplotaxida	Lumbricidae
<i>Aporrectodea caliginosa</i>	Annelida	Haplotaxida	Lumbricidae
<i>Aporrectodea longa</i>	Annelida	Haplotaxida	Lumbricidae
<i>Lumbricus terrestris</i>	Annelida	Haplotaxida	Lumbricidae
<i>Arion intermedius</i>	Gastropoda	Pulmonata	Arionidae
<i>Arion owenii</i>	Gastropoda	Pulmonata	Arionidae
<i>Arion hortensis</i>	Gastropoda	Pulmonata	Arionidae
<i>Arion distinctus</i>	Gastropoda	Pulmonata	Arionidae
<i>Limax flavus</i>	Gastropoda	Pulmonata	Limacidae
<i>Forficula sp.</i>	Arthropoda	Dermaptera	Forficulidae
<i>Erigone dentipalpis</i>	Arthropoda	Araneae	Linyphiidae
<i>Formicidae sp.</i>	Arthropoda	Hymenoptera	Formicidae
<i>Tipulidae sp.</i>	Arthropoda	Diptera	Tipulidae
<i>Notiophilus biguttaus</i>	Arthropoda	Coleoptera	Carabidae
<i>Adalia bipunctata</i>	Arthropoda	Coleoptera	Coccinellidae
<i>Tachyporus obtusus</i>	Arthropoda	Coleoptera	Staphylinidae
<i>Phasmarhabditis hermaphrodita</i>	Nematoda	Rhabditida	Rhabditidae
<i>Steinernema feltiae</i>	Nematoda	Rhabditida	Steinernematidae
<i>Rhabdias spp.</i>	Nematoda	Rhabditida	Rhabdiasidae
<i>Oswaldocruzia filiformis</i>	Nematoda	Ascaridida	Molineidae

**Table 7.2.** Non-target species tested for cross-reactivity with *Neoxysomatium brevicaudatum* primers COI-J-1764 and COI-N-1938.

Primer sensitivity was established with both normal *Taq*, using the conditions described above, and with a Multiplex (Qiagen) kit under the following conditions: 1X Master Mix, 0.2 $\mu$ M each primer and 5ng/ $\mu$ L of DNA with an initial denaturation at 95 $^{\circ}$ C for 15 min, 35 cycles of 94 $^{\circ}$ C for 30 s, 66 $^{\circ}$ C for 90 s and 72 $^{\circ}$ C for 90 s, and a final extension at 72 $^{\circ}$ C for 10 min. Nematode DNA concentrations (determined by Nanodrop ND-1000 Spectrophotometer) were 3, 0.6, 0.3, 0.06, 0.03, 0.006 and 0003  $\mu$ g/ $\mu$ L. Amplification success was determined by electrophoresis. Primers were sensitive up to 0.03  $\mu$ g/ $\mu$ L DNA with normal *Taq* (see Fig. 7.2a) and between 0.003-0.006  $\mu$ g/ $\mu$ L with Multiplex (see Fig. 7.2b).



**Figure 7.2.** Sensitivity of COI-J-297 and COI-N-471 primers amplifying *Neoxysomatium brevicaudatum* DNA. Lanes 1-7: 3, 0.6, 0.3, 0.06, 0.03, 0.006 and 0003  $\mu$ g/ $\mu$ L DNA; lane 8: negative water control; a) without Multiplex, b) with Multiplex.

The accuracy of primer screening was established by comparison with conventional microscopy of slow worm faeces, and gave a 100% detection rate with no false positives (Jones *et. al.*, unpublished).

#### 7.3.4 Faecal screening and statistical analysis

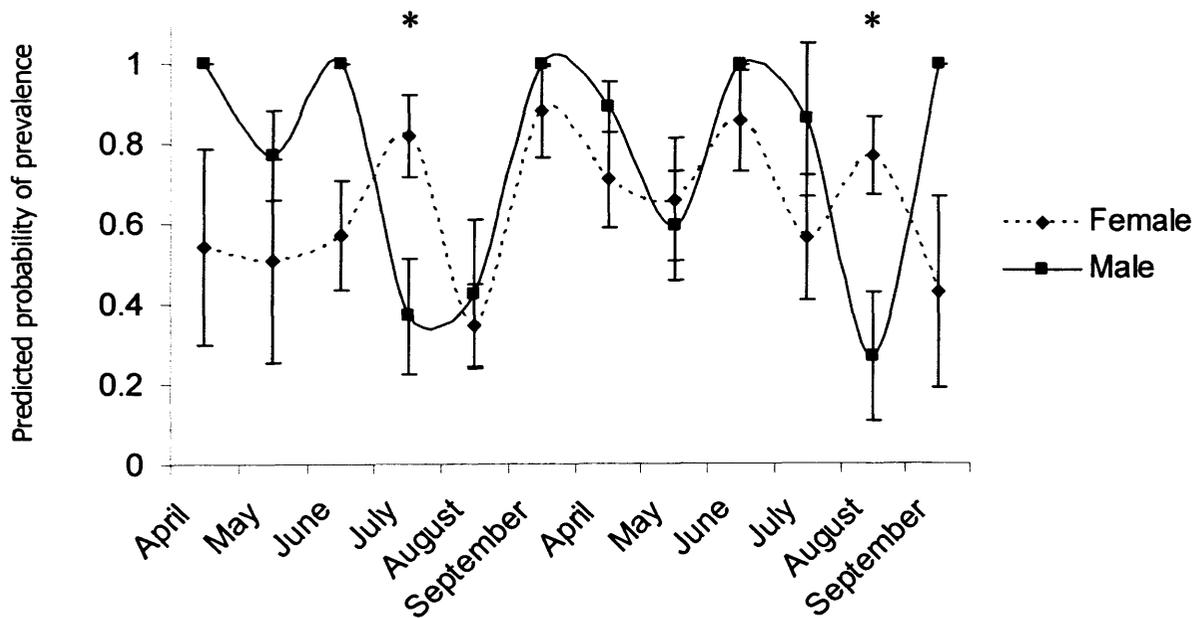
The 270 slow worm faecal samples were extracted using the QIAamp® DNA Stool Mini Kit (Qiagen) in accordance with the manufacturer's instructions. *Neoxysomatium brevicaudatum*-specific primers COI-J-1764 and COI-N-1938 were used with normal *Taq* to amplify DNA from any *N. brevicaudatum* nematodes co-extracted in the faeces. Samples testing negative were rescreened using the Qiagen Multiplex kit. This combined approach was considered more cost-effective than sole use of the more expensive, but more sensitive, Multiplex kit approach.

The effects of slow worm length, weight, age class, sex, and presence/absence of tail, along with site, month, year, temperature (minimum, maximum and mean), rainfall and sunshine on prevalence of *N. brevicaudatum* were explored within a Generalised Linear Model (GLM). Weight, length, temperature, rainfall and sunshine were treated as covariates and all other predictors as factors. Second order interactions included were sex:month, sex:site and length:weight. A binomial error distribution was used with a logit link function. All analyses were conducted in the R statistical package version 2.9.2.

## 7.4 Results

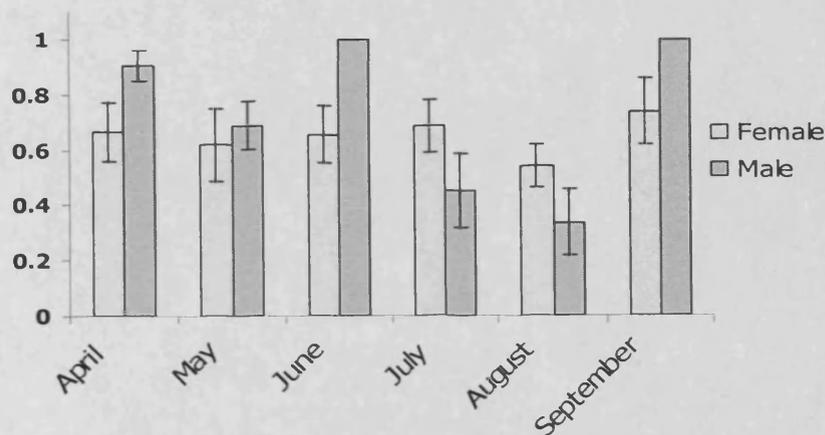
Overall prevalence of *Neoxysomatium brevicaudatum* was 66.4% (95% CI: 57.3-75.5%) and 56.7% (95% CI: 48.7-64.7%) in male and female slow worms respectively, which was not significantly different ( $\chi^2=2.58$ ,  $df=1$ ,  $P=0.108$ ).

Although sex alone did not effect prevalence, the interaction between month and sex was found to have a significant effect ( $\chi^2=15.9$ ,  $df=5$ ,  $P=0.007$ ). Post hoc contrast analysis revealed significantly higher prevalence in males compared to females for April ( $t=1.98$ ,  $df=218$ ,  $p=0.05$ ) (Fig. 7.3). Additionally, prevalence in males in April was significantly higher than it was in July ( $t=2.72$ ,  $df=218$ ,  $p=0.007$ ) and August ( $t=3.32$ ,  $df=218$ ,  $p=0.001$ ). Conversely, prevalence in females did not change from month to month.

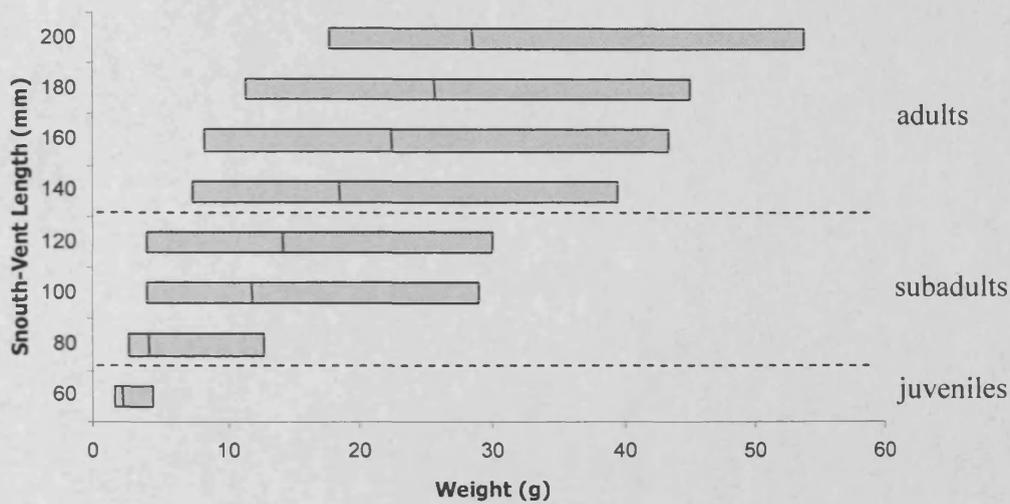


**Figure 7.3.** Predicted probability of *Neoxysomatium brevicaudatum* prevalence (with SE bars) in male and female slow worms showing significantly lower prevalence in males than in females in August 2007 ( $p=0.02$ ) and July 2008 ( $p=0.03$ ).

With 2007 and 2008 analysed separately, the interaction between month and sex was still significant ( $\chi^2=29.7$ ,  $df=11$ ,  $p=0.002$ ), however, due perhaps to the reduced power of the analysis, there was no longer a significant difference in prevalence between males and females in April for either year. Instead, post hoc contrast analysis revealed significantly lower predicted prevalence in males compared to females in mid-summer (July 2007 ( $t=2.34$ ,  $df=204$ ,  $p=0.03$ ) and August 2008 ( $t=2.24$ ,  $df=224$ ,  $p=0.02$ ), Fig. 7.4). This effect was likely masked when 2007 and 2008 were pooled by the decrease in male prevalence happening in different summer months in each of the years.



**Figure 7.4.** Predicted probability of *Neoxysomatium brevicaudatum* prevalence (with SE bars) in male and female slow worms with months pooled for 2007 and 2008, showing significantly higher prevalence in males than in females in April ( $p=0.05$ ).



**Table 7.3.** Weight range (including means) for the different length classes of slow worms (*Anguis fragilis*) found in this study ( $n=270$ ).

An interaction between Length and Weight also had a significant association with the prevalence of nematodes ( $\chi^2=9.7$ ,  $df=1$ ,  $p=0.002$ ). Slow worm size affected the predicted prevalence such that in smaller animals (juveniles and sub-adults, see Table 7.3 for weight ranges found for each size class) there was a positive relationship between prevalence and weight which switched to a negative relationship in larger animals (adults, Fig 5). By using slow worm length as a surrogate for age, and controlling for weight, there is a clear curvilinear pattern of predicted prevalence increasing with age up until adulthood and then decreasing (Fig. 7.6).

No other terms were found to be significant and were removed from the final model.

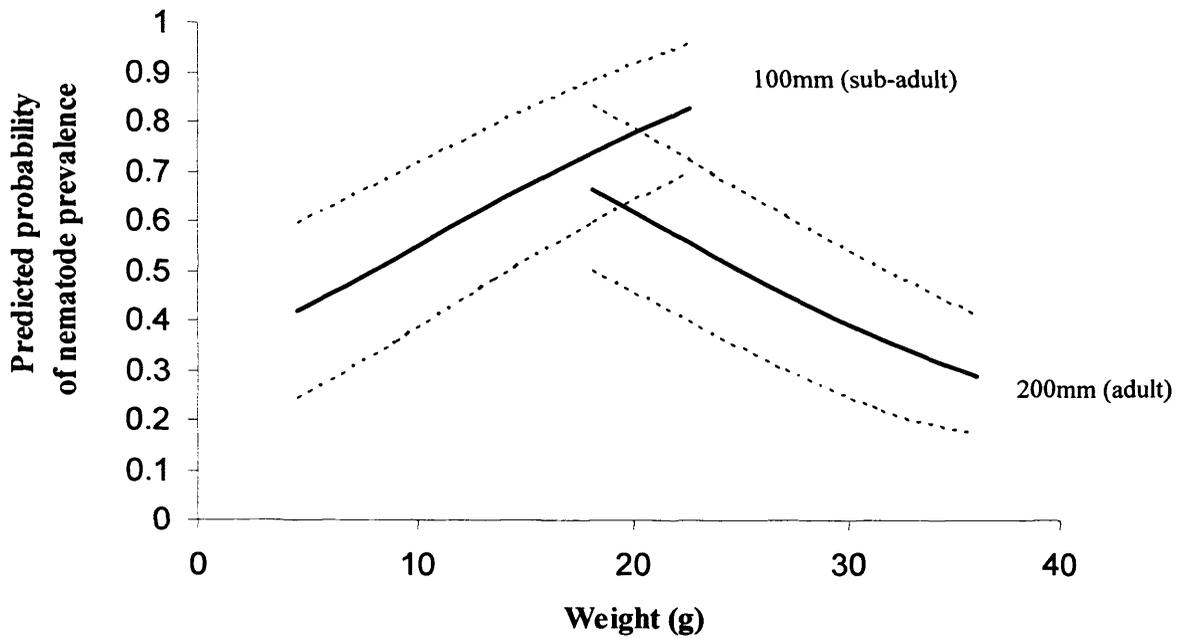


Figure 7.5. Effect of the interaction between slow worm length and weight on the predicted probability of *Neoxysomatium brevicaudatum* prevalence (with SE bars), as found significant in GLM ( $p=0.0007$ ).

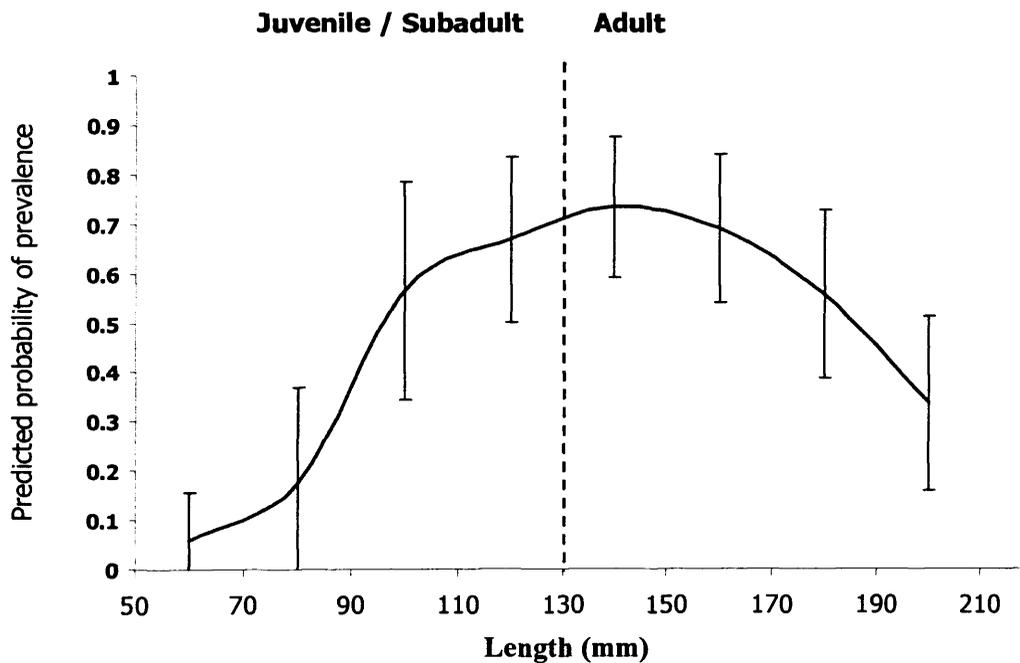


Figure 7.6. Changes in the predicted probability of *Neoxysomatium brevicaudatum* prevalence in slow worms with host length (with SE bars), used as a surrogate for host age. Data shown is calculated for males in May 2008, using average male weight for that month, and is representative of females and of other months.

## 7.5 Discussion

### 7.5.1 Sex bias

The overall prevalence of *Neoxysomatium brevicaudatum* was 66.4% for males and 56.7% for females, which was not significantly different. The GLM revealed that there was a significant effect of sex on parasitic prevalence, but that this sex bias was highly dependent upon month (see Table 7.2). Prevalence in males was significantly higher than in females in April (Fig. 7.3). Differences in parasite prevalence between sexes is generally considered a result of either differential exposure, often arising from behavioural differences (Drobney *et al.* 1983), or in susceptibility, due to differences in either the host's resistance to parasites or their ability to remove them (Zuk 1990). Prevalence of infection is often higher in males than in females in vertebrates of all classes (fish (e.g., Reimchen & Nosil 2001), amphibians (e.g., Dare & Forbes 2008), birds (e.g., Robinson *et al.* 2008), mammals (e.g., Krasnov 2005) and reptiles (e.g., Amo, Lopez & Martin 2005)) by a wide variety of parasites (e.g. protozoan (e.g., Amo, Lopez & Martin 2005), nematodes (e.g., Dare & Forbes 2008), trematodes (e.g., Robinson *et al.* 2008) and mites (e.g., Christe *et al.* 2007)). With the rise in prevalence in males coinciding with the onset of the mating season, when testosterone levels are at their highest in reptiles (e.g., Watt *et al.* 2003; Wack *et al.* 2008), it may be that testosterone-induced immunosuppression, and the resulting increased susceptibility to parasites, is driving higher infection in males.

With the two years analysed separately, prevalence in males was no longer significantly higher than that in females in April for either year, due perhaps to the decreased power of the analysis. Instead, male prevalence was found to be significantly lower than female prevalence in mid-summer of each year, in both July 2007 and August 2008 (Fig.

7.4). This result appears to have been masked in the combined analysis for both years by the trend having happened in a different month each year. This difference in prevalence between the sexes could be attributed to a disparity in habitat use by males and females, or increasing/decreasing encounter rates with parasites. Both sexes share territories throughout the year and are often found together, but this does not necessarily dictate similar foraging behaviour or use of their territory.

### 7.5.2 *Host Body Condition*

There was a significant association between predicted nematode prevalence and an interaction between slow worm length and weight. In slow worms over 150mm (defined as adults), lower weight (i.e. poorer condition) correlated with an increase in predicted prevalence (Fig. 7.5), suggesting *N. brevicaudatum* has a negative effect on slow worm growth and health. This is to be expected and has been reported for a range of nematode-infected hosts (e.g. Calvete *et al.* 2004; Irvine *et al.*, 2006). Alternatively, slow worms with poorer body condition may be more prone to parasites, either through exposure or susceptibility. Curiously, in slow worms below 150mm (i.e. juveniles, sub-adults and young adults) the reverse trend was found: for any given length prevalence increased with an increase in weight, that is, prevalence was positively associated with condition. It may be that in these younger animals, those with the greatest foraging success are increasing not only their intake of food but also their exposure to parasites from accidental consumption. Conversely, it may be that healthier slow worms are simply better able to withstand parasitic infection, while those already in poor condition die as a result of infection and were therefore undetected.

### 7.5.3 *Acquired Immunity*

With length taken as a surrogate for age, and controlling for weight, the prevalence pattern found for *N. brevicaudatum* (Fig. 7.6) shows a positive correlation with age in young individuals followed by a negative correlation in older animals. The initial rise in prevalence is characteristic of the majority of age-intensity and age-prevalence curves, which show a rapid accumulation of parasites with increasing exposure after the age at which an animal is first susceptible to infection (e.g., Hudson 1992; Quinnell 1992; Krasnov *et al.* 2006). Typically such curves reach an asymptote where infection is balanced by parasite mortality. The subsequent decrease in prevalence with age is also common (e.g., Sreter *et al.* 1995; Ladeia-Andrade *et al.* 2009; Tariq *et al.* 2010), and may be attributed to:

1. age-related changes in exposure to parasites, such as changes in host behaviour (e.g. Dalton and Pole 1978; Tinsley 1989) or diet (e.g., Thomas 1965; Borgstrom 1970; Martin *et al.* 2005);

2. age-related changes in susceptibility (reducing parasite establishment, survival or fecundity), either resulting from innate changes in immunity (e.g., Sreter *et al.* 1995; Robb & Forbes 2006) or immunity acquired through repeated exposure (e.g., Folstad *et al.* 1989; Sreter *et al.* 1995; Ladeia-Andrade *et al.* 2009; Wilkins *et al.* 1984, 1987; Crombie and Anderson, 1985; Hagan *et al.* 1991; Kabatereine *et al.* 1999; Faulkner *et al.* 2002); or

3. a combination of both (Anderson 1986).

Evidence for acquired immunity can be determined by identifying and comparing 'peak shift' (Woolhouse 1998) in populations with different transmission rates of exposure, that is, the difference in the age at which intensity peaks between populations (reviewed in

Woolhouse 1998; Wilson et al 2002). A population with a higher transmission will peak earlier, as younger animals experience sufficient exposure to develop immunity.

As prevalence, and not intensity, was measured in this study, identifying any peak shifts between different slow worm populations would not have been possible and so, while the age-prevalence curve found for *N. brevicaudatum* in slow worms may indicate acquired immunity, it may also represent changes in exposure with age. Slow worms of all ages/sizes were found sharing territories and there is no obvious reason to suppose prey-switching with age since the main component of their diet, earthworms and slugs (Luiselli 1992), are accessible to slow worms of all sizes and therefore prey size is unlikely to be a limiting factor. However, if for example nematodes are only present in prey of a certain size (e.g. only in small secondary hosts) then larger slow worms feeding on larger individuals would be expected to have reduced exposure to them. Patterns of prevalence do not necessarily match that of intensity, and hence age-prevalence curves are potentially more ambiguous than those of intensity as any asymptote or reduction of prevalence in the age-prevalence curve could merely indicate the loss of infected individuals from the population. While less powerful, prevalence data is a useful tool for studying epidemiological processes, particularly as it is more readily obtainable using non-invasive techniques such as faecal analysis, which is especially important when examining patterns of parasitism in protected species.

Here we used a non-invasive molecular approach to model, for the first time, parasite prevalence in slow worms, a widespread and locally abundant protected British reptile. We found that prevalence changes with age in a characteristic convex pattern and that there is an association between slow worm condition and nematode prevalence. Furthermore, these

results highlight the importance of considering multiple factors when investigating parasitic infection patterns and exploring sex biases. Sex-biases were identified but sampling in any single month would have lead to unrepresentative conclusions being drawn and as such any studies into sex bias not considering additional variables, such as season and host age / size, should be interpreted with caution.

## 7.6 References

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## **Chapter 8**

### **Molecular detection of the ontogenetic changes in the diet of smooth snakes (*Coronella austriaca*).**

(To be submitted to Conservation Biology)

## 8.1 Abstract

Smooth snakes (*Coronella austriaca*) have a highly restricted distribution in the UK, the reason for which is not understood. Grass snakes (*Natrix natrix*), a sympatric and similar colubrid snake, are common throughout England and Wales. Both species are widespread in continental Europe.

The distribution of a predator will often reflect that of its prey. Therefore understanding an animal's distribution requires a detailed knowledge of its diet. Conventional methods for establishing diet often rely on visual recognition of morphologically identifiable features of prey in faeces, regurgitates or stomach contents which suffer from biases and poor resolution of taxa. DNA-based techniques have allowed for the non-invasive analysis of diet from faeces without these constraints.

Field sites in Dorset and Hampshire were visited each month (Apr-Sept) over two years and faecal samples were collected from smooth snakes and grass snakes. Faecal DNA was screened with species-specific primers for amphibians, reptiles, small mammals and invertebrates.

Over 85% of smooth snakes were found to have eaten reptiles and 28% had eaten small mammals. Predation on small mammals increased with age in adults and was entirely absent in the diet of juveniles and sub-adults. Predation on reptiles did not change ontogenetically, indicating that juvenile snakes may be restricted to a reptile diet. Grass snakes were less likely to consume small mammals (14%) but more likely to consume amphibians (64% compared to 5% in smooth snakes). Predation on reptiles, however, was much higher than previously thought (69%). This suggests that there may be competition between grass snakes and smooth snakes at a juvenile stage, which may be a factor

influencing the restricted distribution of smooth snakes to areas of sufficiently high reptile densities to support both species.

These findings are of importance to reintroduction and conservation management strategies, which require information that will lead on to strategies that will provide the conditions necessary for maintaining higher densities of reptiles.

## 8.2 Introduction

The long term coexistence of competitive species has often been described as impossible (Volterra 1928; MacArthur and Levins 1964; Levins 1968; Rescigno and Richardson 1965; Levin 1970) and according to Gause's "law of competitive exclusion" (Gause 1934) and Hardin's "competitive exclusion principle" (Hardin 1960) two consumer species competing for the same resources cannot coexist if all other ecological factors are equal. If both species are specialised on the same resource, whichever species has even the slightest advantage will always dominate in the long run, resulting in either the competing species' local extinction (Moulton and Pimm 1986), displacement (MacArthur 1972; May 1974; Schoener 1974; Ioue *et al.* 2008) or shift in dietary specialization (Arlettaz *et al.* 1997). Where consumers share multiple resources, coexistence is possible only if these resources are partitioned differently among themselves (Schoener 1974) or if the effect one species has on the other (interspecific competition) is greater than the effect it has on individuals of its own species (intraspecific competition) (MacArthur 1970; Tilman 1982).

Prey availability is one of the key mechanisms driving predator distribution. Although the distributions of snakes in temperate regions may be strongly influenced by temperature and the ability to thermoregulate (Huey 1991; Reinert 1993, Row and Blouin-

Demers 2006), and the presence of winter hibernation sites (Prior and Weatherhead 1996; Harvey and Weatherhead 2006), the “ideal free distribution theory” (Fretwell and Lucas 1970; Fretwell 1972) predicts that the distribution of any predator will reflect that of its prey, and that this is most often the driving factor. The home ranges of black pine snakes (*Pituophis melanoleucus lodingi*) (Baxley and Qualls, 2009), water pythons (*Liasis fuscus*) (Madsen and Shine 1996) and carpet pythons (*Morelia spilota metcalfei*) (Heard, Black and Robertson 2004) have all been found to be associated with the abundance of their prey. While the distribution of predators may be restricted to areas of sufficiently high prey density, ontogenetic shifts in diet, a common phenomenon among vertebrates, can mean that a predator’s distribution may be dependent upon the spectrum of different prey available at different stages of its life. Differences between juveniles and adults in their prey, and the size of prey, has been observed in fish (e.g., McCormick 1998; Renones, Polunin and Goni 2002), birds (Price and Grant 1984), mammals (e.g. Page *et al.* 2005; Dickman 1988) and reptiles (Lind and Walsh 1994; Herrel and O’Reilly 2006), and is commonly seen in snakes (e.g. Pizzatto, Marques and Facure 2010). Frequently, juveniles eat smaller prey and a narrower range of them than adults, often considered to be a function of differences in body size, but which can also be attributed to inexperienced foraging ability (eg., Rutz, Whittingham and Newton 2006), or differential habitat use due to changes in predator avoidance or territory defense with age. Specialisation on different developmental stages of the same prey by competing predators, usually a result of differences in feeding strategies or physical limitations, may potentially lead to coexistence. Even so, a predator exploiting one stage is likely to decrease the abundance of later stages and therefore outcompete any predator dependent on these.

In the UK, the smooth snake (*Coronella austriaca*) is considered endangered due to its severely restricted distribution in a few strongholds in Dorset and Hampshire, southern England, the reasons for which are not clear. Britain is home to two other sympatric snakes, the adder (*Vipera berus*) and the grass snake (*Natrix natrix*), both of which are much more widely distributed. The adder's range extends from the southern coast of England to the northern coast of mainland Scotland (Reading *et al.* 1996), whereas the grass snake is rare above 56°N, approximately the border of England and Scotland. With grass snakes mostly absent from areas where the average annual temperature is below 8.4 °C, their distribution is probably a constraint imposed by thermoregulation. The more efficient thermoregulatory behaviour of adders, coupled with a very diverse diet (Prestt 1971; Andren and Nilson 1983; Drobenkov 1995), explains adders' ability to range farther north. It has been suggested, as smooth snakes are only found on sandy lowland heath in England, that this habitat may be structurally or ecologically important to them (Gent 1988; Spellerberg and Phelps 1977; Goddard 1981). The sand lizard (*Lacerta agilis*) is restricted to the same areas due to a requirement for open exposed sand for egg-laying (Corbett and Tamarind 1979). This, and a need for more sunshine than other British reptiles (Dent and Spellerberg 1987), explains the confined distribution of sand lizards in southern Britain. Smooth snakes, conversely, are found in a variety of different habitats throughout continental Europe (pine forests, mixed riverside forests, bogs, roadside verges, vegetation bordering fields, bracken / bramble patches and shrub land, orchards and open grassland (Beebee and Griffiths 2000)), so the basis of any dependency on one habitat type in England is evidently not structural. Smooth snakes range almost as far north as grass snakes, throughout mainland Europe, up into the south of Norway, and so a restriction based on temperature and microhabitat features

beneficial for thermoregulation seems unlikely too. Alternatively, smooth snake distribution may be more ecological, a function of diet, prey availability, prey diversity and competition with sympatric snakes for food (Phelps 1978; Goddard 1984; Drobenkov 1995).

Smooth snakes are generally considered to be reptile specialists throughout continental Europe (Duguy 1961; Bruno 1966; Andren and Nilson 1976, 1979; Street 1979; Drobenkov 1995; Rugiero *et al.* 1995). However, their diet in the UK has been a subject of debate, and while there is agreement over the main range of prey taken (amphibians, reptiles and small mammals) the importance of each is unclear. In a study of the diet of a Lithuanian population of smooth snakes, only five different species of prey were found (determined by dissection and regurgitate analyses), four of which were reptiles and one a small mammal, which comprised just 6.9% of the diet (Drobenkov 1995). Similarly, the diet of smooth snakes in Italy was found to have a low diversity of prey and to be dominated by lizards, which comprised 88.6% of the diet (Rugiero *et al.* 1995). Corbett (in Nature Conservancy Council, 1983) collated various studies of smooth snake diet conducted in the UK which revealed far greater diversity of prey than expected. The report identified at least nine different prey species in UK populations, with 58.3% represented by reptiles and 28.2% by small mammals, with lesser numbers of birds (10.4%) and amphibians (2.1%). These British studies used a variety of different methods for establishing diet: microscopic analysis of faeces and regurgitates, post-mortems and direct observations. Presumably, data based on direct observation is biased towards predation on reptile prey while underestimating that on small mammals, as juvenile mammals are usually in underground burrows. As such, if all observed data is omitted from Corbett's report then mammalian and reptilian prey appear equally important, with both making up 46.4% of the diet. In faecal and regurgitate analyses,

Goddard (1981, 1984) found the proportion of smooth snakes which had consumed small mammals was more than twice that of reptiles. Goddard (1984) speculated that smooth snakes were not reptile specialists, but rather generalists consuming prey in relation to its availability, and that the higher reptile component of their diet in continental Europe simply reflected the higher relative densities of reptiles there. This was supported by Rugeiro *et al.* (1995), whose faecal and regurgitate analyses of smooth snakes in Italy reveal they were consuming lizards, snakes and mice in accordance with their ratio of in the wild. However, studies have revealed an innate feeding preference for lizards in juvenile smooth snakes (Goddard 1984) indicating that smooth snakes may initially be restricted to a reptile diet, which broadens with increasing age, size and experience. At an even younger age, smooth snakes might be restricted to a diet of invertebrates, with a number of reports of invertebrates in their diet (Spellerberg and Phelps 1977; Corbett in Nature Conservancy Council, 1983; Rugiero *et al.* 1995).

The diets of Britain's other native snakes are more firmly established, both in the UK and throughout Europe, with adders found to have an extremely broad diet which includes amphibians, reptiles and birds, but predominantly small mammals (Prestt 1971; Drobenkov 1995), while grass snakes are amphibian specialists that take little other prey (Drobenkov 1995). Although there is more overlap in the diet of adders with both grass snakes and smooth snakes (Drobenkov 1995), the home ranges of adders seldom overlap those of the others snake species (Spellerberg 1977), whereas grass snakes and smooth snakes are frequently found together. As a result, there is greater potential for competition between these two species. Grass snakes will include in their diet reptiles (Luiselli and Rugiero 1991; Capula, Rugiero and Luiselli 1994; Drobenkov 1995; Filippi *et al.* 1996; Luiselli and Capula

1997) and small mammals (Luiselli and Rugiero 1991; Luiselli and Capula 1997; Gregory and Isaac 2004); and smooth snakes have been found to eat amphibians (Corbett in Nature Conservancy Council, 1983), although these are considered small components of each of their diets. However, snake size and age is rarely accounted for in these studies, which have usually been conducted on adults only. As there is evidence of ontogenetic shifts in diet for grass snakes (Gregory and Isaac 2004) and it has been suggested for smooth snakes (Goddard 1981) these studies may well be missing critical information on juveniles diets. If smooth snakes are dependent on a narrow range of specific prey at a juvenile stage, then the abundance and distribution of those prey may place restrictions on their population density and may drive them into competition with grass snakes, adders and other predators.

British reptiles are in decline as habitats are continually destroyed, fragmented or unsympathetically managed, with their ranges increasingly becoming narrower and species having been driven to extinction in many regions (eg. Howes 1973; Prestt 1974). Prestt (1974) suggested that the extinction of some species of reptile in the UK was likely in the near future without continual conservation effort. Appropriate conservation management of endangered species requires a comprehensive understanding of all of the prey important to it throughout its lifetime, in order to better understand its distribution and mitigate against restrictions upon its survival.

Conventional studies of diet, based on the analysis of faeces or regurgitates for morphologically identifiable features of prey, are constrained by the presence of undigested remains and the ability to accurately identify them. Snakes are known to be able to digest prey thoroughly, digesting even bones and other hard parts (Secor 2008). Certainly, if soft-bodied invertebrate prey, such as slugs or earthworms, were included in their diet then

traditional methods would not be able to identify them. Molecular techniques, in particular the detection of prey DNA in faeces (Symondson 2002), has enabled detailed analyses of prey consumed by fish (e.g. Saitoh *et al.* 2003; Jarman and Wilson 2004), birds (e.g. Jarman *et al.* 2004; Deagle *et al.* 2007), and mammals (e.g. Jarman *et al.* 2002, 2004; Marshall *et al.* 2010). These techniques have never been applied to reptile diets before, and if proven successful would open up a new area of ecological study to herpetologists.

In this study we used molecular tools to investigate predation by smooth snakes and grass snakes on various amphibian, reptile, small mammal and invertebrate prey in order to address the following hypotheses: There are ontogenetic changes in the diet of smooth snakes; If smooth snakes are dietary specialists, diet should remain similar in two different habitats despite predictable differences in prey assemblages; There is overlap in the diet of grass snakes and smooth snakes at specific, or all, age / size classes, and therefore the potential for competition between them.

### 8.3 Methods

#### 8.3.1 *Non-target DNA extraction*

All animals used for extraction were donated by small mammal and herpetological groups, having been found dead during animal surveys. Animals collected were: common vole (*Microtus arvalis*), field vole (*Microtus agrestis*), bank vole (*Clethrionomys glareolus*), common shrew (*Sorex araneus*), pygmy shrew (*S. minutus*), water shrew (*Neomys fodiens*), brown rat (*Rattus norvegicus*), yellow necked mouse (*Apodemus flavicollis*), house mouse (*Mus musculus*), palmate newt (*Lissotriton helveticus*), smooth newt (*L. vulgaris*), common lizard (*Lacerta vivipara*), sand lizard (*L. agilis*), slow worm (*Anguis fragilis*), adder (*Vipera*

*berus*), grass snake (*Natrix natrix*) and smooth snake (*Coronella austriaca*). The DNeasy® Tissue Kit (Qiagen) was used for extraction of DNA from tissue. To ensure extraction success, all DNA was amplified in PCR with universal primers LCO1498 (Folmer *et al.* 1994) and C1-N-1770 (see Section 2.3.3.2 for primers' development) with the following conditions: 1X buffer, 2 mM MgCl<sub>2</sub>, 0.5 mM dNTP (Invitrogen), 0.5 μM of each primer, 0.38 U *Taq* polymerase (Invitrogen) and 2 μL/ 25 μL of DNA with an initial denaturation at 94 °C for 3 min, 35 cycles of 94 °C for 30 s, 48 °C for 30 s and 72 °C for 45 s, and a final extension at 72 °C for 5 min. Amplification was visualized by gel electrophoresis stained with ethidium bromide. Double-distilled water was included as a negative control to test for contamination.

PCR products were sequenced for species for which sequences were not readily available on Genbank (slow worm, common lizard, smooth snake, grass snake and adder). They were cleaned using ExoSAP in the following reaction: 10 μL of each PCR product, 0.25 μL Exonuclease I, 0.5 μL SAP (shrimp alkaline phosphatase) and incubated for 45 min at 37°C and 15 min at 80°C. Cleaned product was then used in sequencing PCR using a Big Dye™ terminator sequencing kit (Promega, Madison, WI, USA). Sequences were checked for errors using Sequencher 3.1.2.

### 8.3.2 *Species-specific primer design*

Species-specific primers for common lizard, slow worm, smooth newt and common frog were designed (Figs 8.1 for reptiles and 8.2 for amphibians). Mitochondrial cytochrome *b* sequences for common frog (Accession no. FJ030872.1), palmate newt (*vulgaris* U55948.1), smooth newt (DQ821238.1) and red-spotted toad (*Bufo punctatus*, DQ085775.1), as there

was no homologous sequence for common toad, were downloaded from Genbank. Alignments of sequences are shown in Figures 8.1 and 8.2. A house mouse specific primer was designed, described in Section 2.3.2. Other primers used included bank vole specific- (BV-CG95 and BV-CG266), common shrew specific- (SA520 and SA628), and pygmy shrew specific- (SM421 and SM544) primers (Moran *et al.* 2008), general earthworm primers (185F and 14233R) (Harper *et al.* 2005) and *Arion*-specific primers (Harper *et al.* 2005).

### 8.3.3 *Field sites and faecal collection*

Details of the two field sites (Ringwood and Wareham) used in this study, along with details of faecal collection methodology, are described in Section 2.6.1. Faecal samples from 58 smooth snakes and 14 grass snakes were collected.

LCO1498

**GGTCAACAA ATCATAAAGA TATTGG** →

i) TGGTCAACAA ATCATAAAGA TATTGGAACC CTCTACCTAA TTTTGGGTGC GTGGGCCGGC  
ii) ----- --CATAAAGA TATTGGCACC CTATATTTAC TATTTGGTGC CTGGGCTGGC  
iii) TGGTCAACAA ATCATAAAGA TATTGGAACC CTATACCTAC TATTTGGTGC GTGGTCGGGT  
iv) TGGTCAACAA ATCATAAAGA TATTGGAACC CTTTATCTAC TATTTGGGGC CTGATCCGGA  
v) TGGTCAACAA ATCATAAAGA TATTGGAACC CTATACTTTA TATTCGGCGC CTGATCCGGA

*Anguis fragilis* (AF-110-R)

← CGCAGAGT TAAGCCAGCC

i) ATGGTGGGAA CCGCCCTAAG CCTGCTAATT CG..... CGGAGCCCTC  
ii) ATAGTAGGTA CAGCTCTTAG TCTTTTAATT CGAA.C..AC .....A.. TGGTACACTT  
iii) TTAGTTGGGG CCTGCCTAAG CATCCTAATG CGAAT....C .G.C..... GGGCTCATTG  
iv) CTAATCGGAG CTTGTCTAAG CATTCTAATA CGAAT...AC .C.CA..... CGGATCCCTA  
v) CTAGTCGGAG CCTGCCTAAG CATCTTAATG CGAAT...AC .C.CT..... AGGGTCCCTA

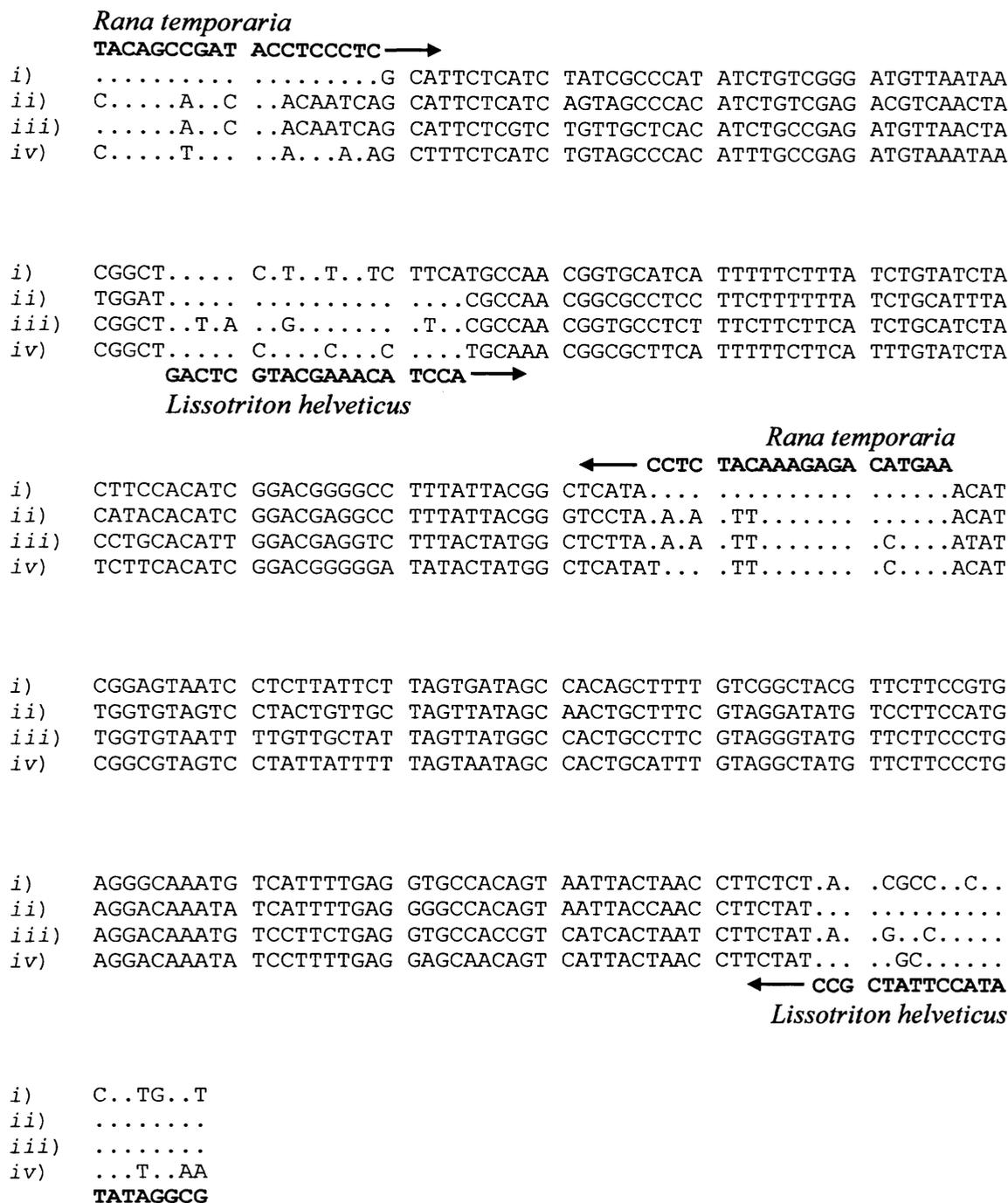
i) CTTGGAGACG ATCAAATCTA TAACGTAATT GTTACTGCC ATGCTTTTGT CATAATTTTC  
ii) CTTGGAGATG ATCAGGTTTA CAATGTTGTT GTTACAGCCC ATGCTTTTGT CATAATTTTC  
iii) CTAGGCAGCG ACCAGATTTT TAATGTTCTA GTTACAGCCC ATGCATTTAT CATAATTTTC  
iv) TTCGGAAGTG ACCAGATCTT TAATGTCCTA GTCACAGCCC ACGCATTTCAT TATAANTTTC  
v) TTTGGCAGCG ATCAGATCTT TAATGTCCTA GTAACCGCAC ACGCCTTCAT CATAATCTTC

*Lacerta vivipara* (LV-216-R)

← GTAAT AATTGGTGGG TTCGGG

i) TTTATGGTAA TACCTA.T.. G..C..C..A TTCGGA  
ii) TTCTTAGTAA TACCT..... TTCGGG  
iii) TTCATAGTAA TACCTA.T.. .....C..C TTCGGA  
iv) TTCATAGTAA TACCTA.T.. .....A..C TTCGGC  
v) TTTATGGTTA TACCGA.T.. ...C..G..C TTTGGA

**Figure 8.1.** Alignment of reptile cytochrome oxidase I sequences, showing position of shared forward primer LCO1498 (Folmer *et al.* 1994) and the reverse complement of the reverse primers, specific for *Anguis fragilis* and *Lacerta vivipara*. (.) indicates base matches with the primer. (i) *Anguis fragilis*, (ii) *Lacerta vivipara*, (iii) *Coronella austriaca*, (iv) *Natrix natrix*, (v) *Vipera berus*.



**Figure 8.2.** Alignment of amphibian cytochrome *b* sequences, showing position of forward primers and the reverse complement of the reverse primers, specific for *Rana temporaria* and *Lissotriton helveticus*. (.) indicates base matches with the primer. (i) *Rana temporaria*, (ii) *Triturus vulgaris*, (iii) *Lissotriton helveticus*, (iv) *Bufo punctatus*.

#### 8.3.4 *Primer optimization and screening*

A temperature gradient PCR was performed for each primer set using DNA of the target, with annealing temperatures ranging from 50-65 °C, to determine the highest temperature a primer pair would amplify the target. PCR was performed using a Peltier Thermal Cycler (Bio-Rad Laboratories, CA, USA). PCR concentrations used were the same as those detailed in Section 10.2.1, but with a PCR cycle of 94 °C for 3 min, 35 cycles of 94 °C for 30 s, the highest working annealing temperature for that primer pair for 45 s and 68 °C for 45 s, and a final extension at 68 °C for 10 min. Amplification was visualized by gel electrophoresis stained with ethidium bromide, and double-distilled water was included as a negative control to test for contamination.

Species-specificity was achieved for common shrew (using an annealing temperature of 64 °C), smooth newt (at 55.5 °C), common lizard (at 62 °C), slow worm (at 54 °C), and group specificity for yellow necked mice (at 62 °C), earthworm (at 65 °C) and *Arion* slugs (at 57 °C). The bank vole primers cross-amplified with field vole at all temperatures, but with no other taxa at 58 °C. The pygmy shrew primers cross-amplified with common shrew and water shrew at all temperatures, but were specific to all three (i.e. general shrew-specific) at 53 °C. The common shrew primers, between 52 °C and 64 °C, resulted serendipitously in bands that were species-specific in pygmy shrews (with an approx. 150 base pair fragment) and water shrew (with an approx. 250 base pair fragment), both distinguishable from the approx. 200 bp fragment for common shrew. These may be the result of amplification of pseudogenes, but as they proved to be species-specific they were considered as suitably diagnostic to be used to identify all three species of shrew in snake faeces. The common lizard primers cross-amplified with sand lizard between 53 °C and 62

°C, and were used as general lacertid primers at 53 °C. The house mouse primers cross-amplified with all small mammals at all temperatures, and were used at 55.5 °C as a general small mammal primer.

All faecal samples were screened with each primer pair twice, and any bands determined by gel electrophoresis scored. Tissue DNA was included as a positive control, to ensure PCR success, and water was included as a negative control to check for contamination.

### **8.3.5** *Statistics*

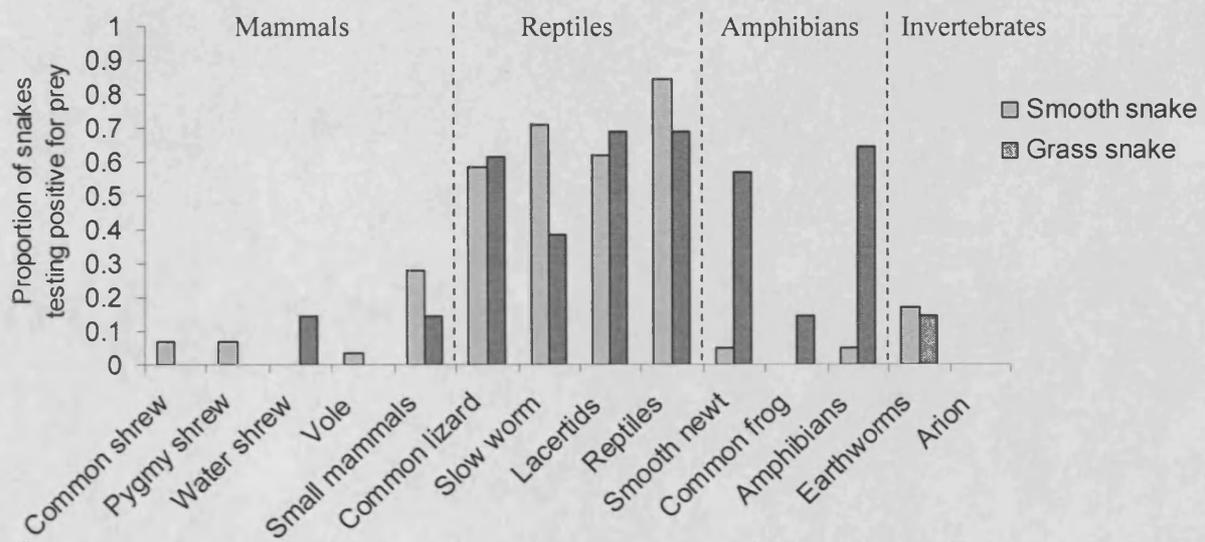
The effects of smooth snake length, weight and sex, along with site, month, temperature, rainfall and sunshine on predation of various prey were explored within a Generalised Linear Model (GLM). Weight, length, temperature, rainfall and sunshine were treated as covariates and all other predictors as factors. The effects of grass snake length, only, were considered within GLMs investigating their predation on prey. A binomial error distribution was used with a logit link function. All analyses were conducted in the R statistical package version 2.8.2.

## **8.4** **Results**

### **8.4.1** *Comparison of smooth snake and grass snake diet*

Overall predation on small mammals by smooth snakes was twice that of grass snakes. The range of small mammals eaten by smooth snakes was wider and non-overlapping with those eaten by grass snakes; smooth snakes consumed common shrews, pygmy shrews and voles, whereas grass snakes were only found to have eaten water shrew (Fig. 8.3). There was no

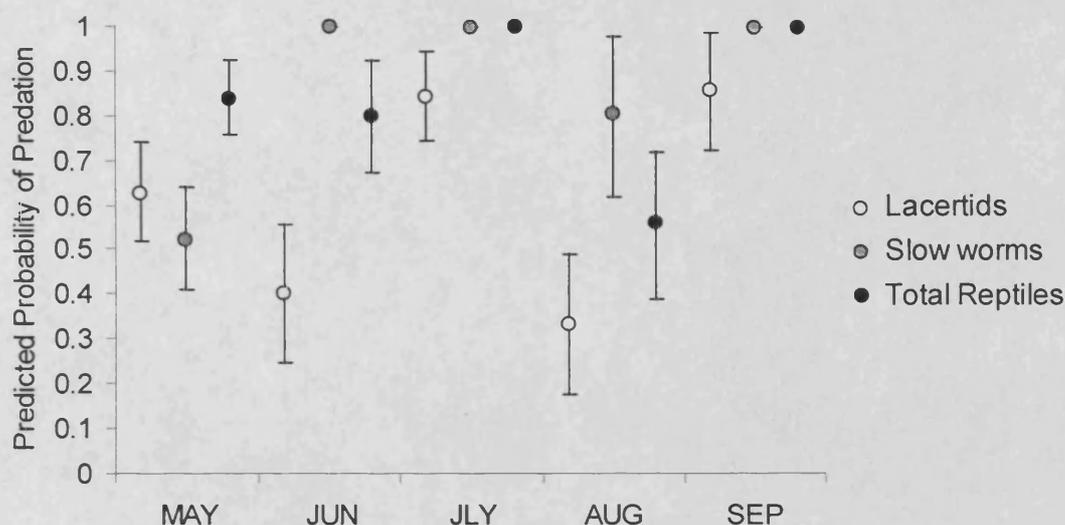
significant difference in predation by the two snake species on common lizards and lacertids (common lizards and sand lizards combined), but predation on slow worms was significantly higher in smooth snakes ( $\chi^2=5.98$ ,  $df=1$ ,  $P=0.014$ ). Predation on amphibians (in particular smooth newts) was over ten times as high in grass snakes as in smooth snakes.



**Figure 8.3.** Proportion of smooth snakes ( $n=58$ ) and grass snakes ( $n=14$ ) testing positive for different mammal, reptiles, amphibian and invertebrate prey using specific primers in PCR.

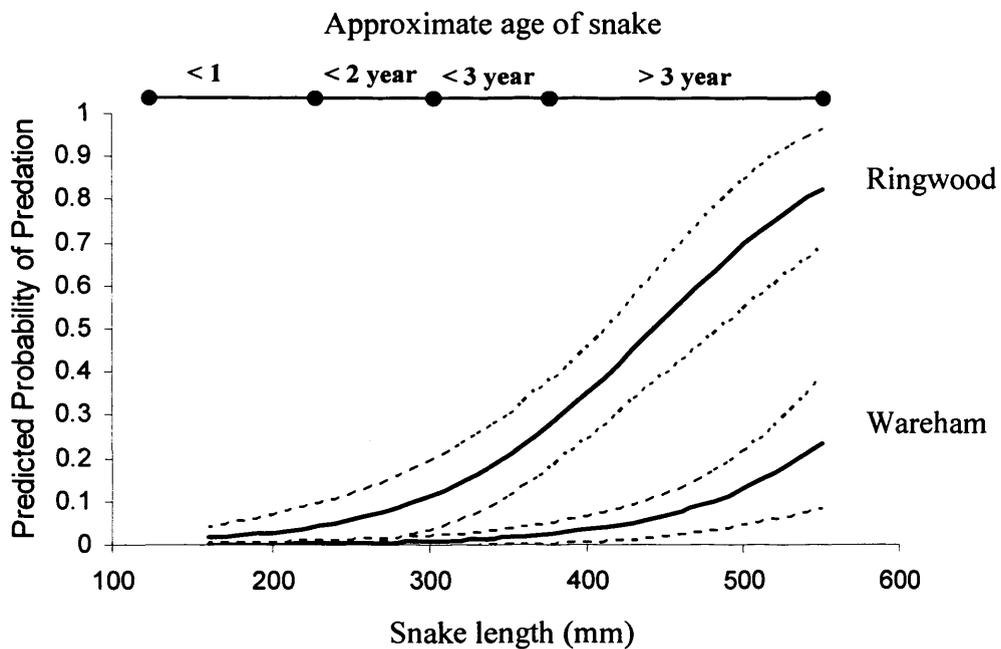
#### 8.4.2 Predation by smooth snakes

There was a significant effect of month on smooth snake predation on slow worms ( $\chi^2=18.3$ ,  $df=4$ ,  $P=0.001$ ), lacertids ( $\chi^2=10.2$ ,  $df=4$ ,  $P=0.038$ ) and on all lizards combined ( $\chi^2=11.1$ ,  $df=4$ ,  $P=0.025$ ), but not when common lizards were considered on their own. Predation on reptiles was high throughout the active season (Fig. 8.4). Even in August, when predation on reptiles was at its lowest, it was still above 50%.

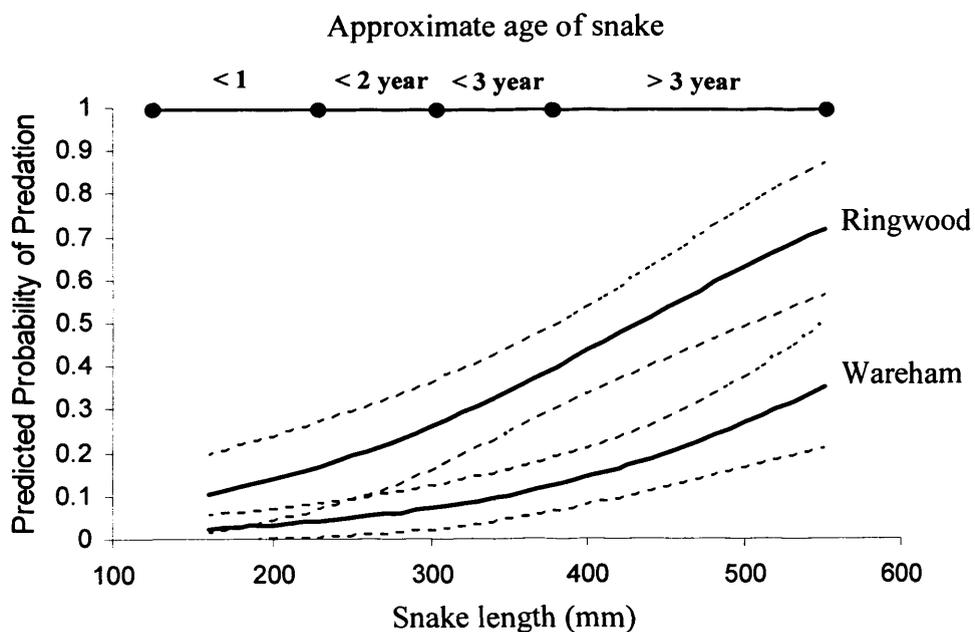


**Figure 8.4.** Predicted probability of predation on lacertids, slow worms (*Anguis fragilis*) and total lizards by smooth snakes (with SE bars), showing significantly different predation in relation to month (determined in GLM). Due to the lack of variation in the data for slow worms and all reptiles combined, individual significant differences cannot be calculated.

There was a significant affect of both snake length and site on predation of shrews by smooth snakes, with predation increasing with snake size ( $\chi^2=10.4$ ,  $df=1$ ,  $P=0.003$ , Fig. 8.5) and much higher predation at Ringwood than at Wareham ( $\chi^2=8.8$ ,  $df=1$ ,  $P=0.001$ , Fig. 8.5). The same effect of length and site were seen when predation on all small mammals combined was analysed (length:  $\chi^2=5.5$ ,  $df=1$ ,  $P=0.020$ ; site:  $\chi^2=5.0$ ,  $df=1$ ,  $P=0.026$ , Fig. 8.6)



**Figure 8.5.** Predicted probability of predation on shrews (common and pygmy) by smooth snakes (with SE, dotted line), showing significant difference between sites and a significant effect of snake length (determined by GLM).

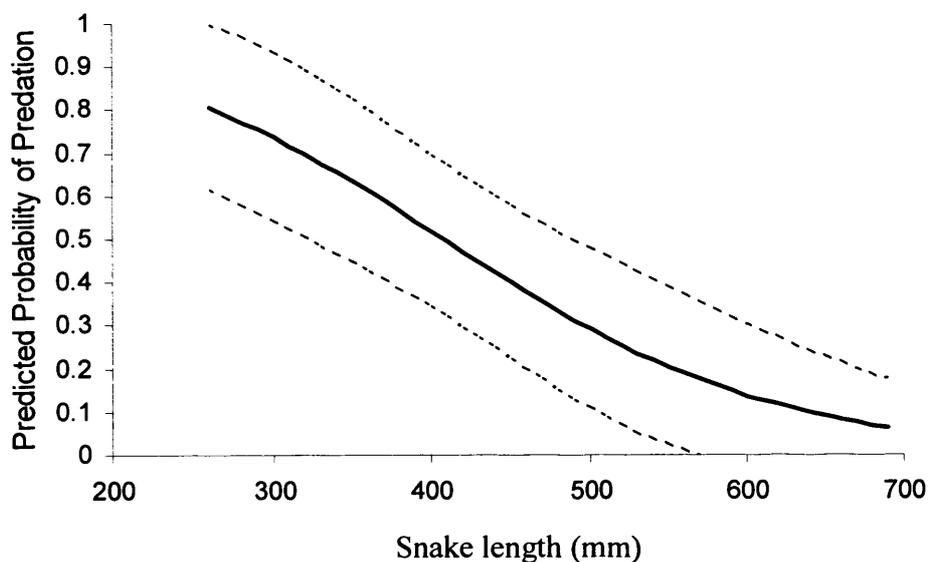


**Figure 8.6.** Predicted probability of predation on small mammals by smooth snakes (with SE, dotted line), showing significant difference between sites and a significant effect of snake length (determined by GLM).

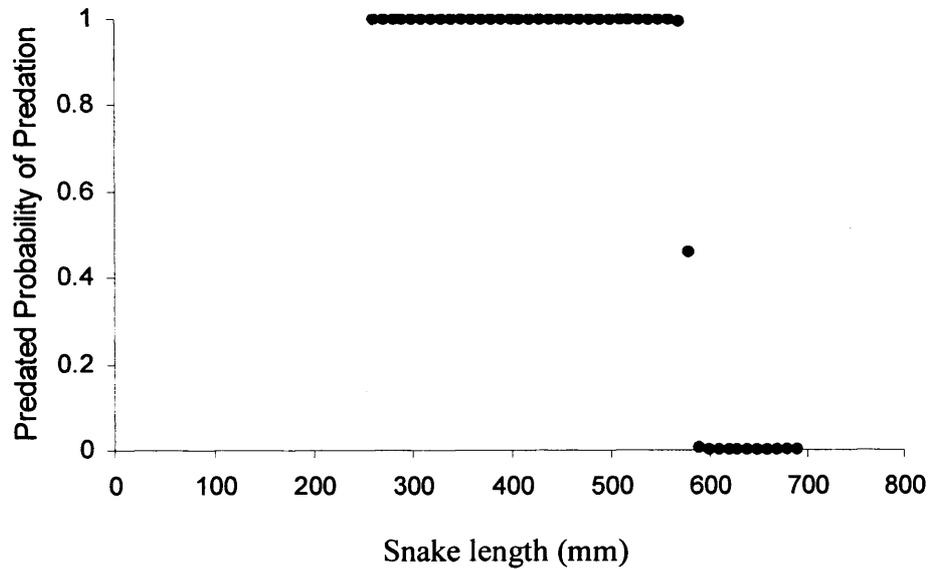
There was no significant effect on earthworm predation of any of the variables considered, implying they were consumed equally regardless of site, sex or length / age. Predation on smooth newts and common frogs was too low to explore statistically.

#### 8.4.3 Predation by grass snakes

Predation by grass snakes on slow worms appeared to be negatively affected by snake length, although this trend was not quite significant ( $\chi^2=17.1$ ,  $df=1$ ,  $P=0.078$ , Fig. 8.7). There were no effect of length on common lizard or lacertid predation, however predation on reptiles overall was highly negatively significantly affected by length (length:  $\chi^2=10.4$ ,  $df=1$ ,  $P=0.001$ , Fig. 8.8).



**Figure 8.7.** Predicted probability of predation on slow worms by grass snakes (with SE, dotted line), showing a *non-significant* negative trend with increasing snake length (determined by GLM).



**Figure 8.8.** Predicted probability of predation on all reptiles by grass snakes (with SE, dotted line), showing a negative trend with increasing snake length (determined by GLM).

There was no effect of snake length on newt predation. All other prey (small mammals, common frog and earthworm) was predated on too low for statistical analysis.

## 8.5 Discussion

This study showed, as in previous studies, high rates of predation on reptiles by smooth snakes (84.5%) but also demonstrated that small mammals were a common prey (28.0%). Due to differences between primers in efficiency it is not appropriate to statistically compare these values with one another. However, it is clear that lizards (lacertids and slow worms) are major components of smooth snake diet.

Predation on small mammals by smooth snakes differed between the two sites, being over twice as high at Ringwood (38.3%) as at Wareham (16.7%). Presumably this reflects differences in prey availability at the two locations rather than behavioural differences

between snake populations. The Ringwood site has a variety of different habitats in close proximity to the heathland, including grassland and forest, which are likely to support more small mammals than the open heathland of Wareham. Predation on reptiles between the two sites did not significantly differ, with 85.7% of snakes at Ringwood and 83.3% at Wareham having consumed them. This indicates that small mammals are not an essential part of smooth snake diet, but are taken in accordance with their availability, as suggested by Goddard (1984) and Rugiero *et al.* (1995). Reptiles, however, appear predominant in their diet regardless of the availability of alternative prey, suggesting a preference or specialism on them. Only two contrasting habitats were considered in this study though, so inferences about differences between them must be interpreted cautiously. Goddard (1984) found no differences in smooth snake diet between UK sites, although the habitats of the three sites considered were all similar to each other and to the Ringwood site of this study. In keeping with this, he found high levels of predation on small mammals.

Smooth snakes showed increased predation on shrews ( $p=0.003$ ) and small mammals ( $p=0.02$ ) with increasing snake size. Taking length as a proxy for age, this indicates an ontogenetic shift in smooth snake diet, with them not taking any small mammals at a young age and increasingly preying on them as they grow, either because of initial preferences for reptile prey or an inability to find, handle or consume small mammals when young. No smooth snakes below 300mm in length were found to have consumed any small mammals, equating approximately to a three year old snake (Goddard 1984), so in these first few years their diet was likely to have been almost exclusively reptile. There was no change in predation on any reptiles (common lizard, lacertids generally or slow worm) with snake size / age, with predation on them starting when smooth snakes were as little as 190mm in length,

within their first year. Most probably, the youngest smooth snakes are eating juvenile lizards. They continue eating lizards throughout their life, while incorporating small mammals as they grow larger / older.

If their distribution is restricted by prey availability, then it is most likely that it is at a juvenile stage, where their diet is at its narrowest and they are predominantly dependent on juvenile lizards. While smooth snakes are clearly capable of eating invertebrate prey, only 17% were found to have consumed earthworm, and juveniles were no more likely to consume them than adults. No snakes were found to have consumed any *Arion* slug. Based on tongue-flick experiments, Pernetta, Reading and Allen (2009) found that smooth snakes showed a preference for lizard and mammal prey over invertebrates, even as juveniles. Bund (1964) and Spellerberg (1977) both suggested that the narrow food preference of young smooth snakes make them particularly vulnerable, more so than grass snakes and adders which have more diverse diets (Drobenkov 1995). Slow worms and common lizards are ubiquitous throughout the UK, and so the distribution of smooth snakes would be expected to be more widespread if it were primarily determined by the distribution of lizard prey. However, it may be that smooth snakes are restricted not just to areas where lizards are present, but to areas with a sufficiently high density of juvenile lizards. The heaths of southern England have higher densities of common lizards, sand lizards and slow worms than elsewhere in the country (Braithwaite *et al.* 1989).

Predation by grass snakes on small mammals and amphibians was as expected based on previous studies (Drobenkov 1995; Gregory and Isaac 2005), with predation on small mammals just half that of predation by smooth snakes, 14.3% compared to 28.0%, while predation on amphibians (smooth newt and common frog) was much higher, 64.3%

compared to 5.2%. However, grass snakes were found to be consuming far greater numbers of reptile prey (68.2%) than previous studies have found (Drobenkov 1995; Gregory and Isaac 2005). There was no significant difference between consumption of common lizards by grass snakes and smooth snakes, indicating the potential for competition between these species. Predation on slow worms appeared to decrease with grass snake length / age (although the trend was not significant). With all reptile prey considered together, the trend was strong and significant ( $p=0.001$ ). There is no obvious explanation for such a decline, but it would explain why most prior studies do not identify reptiles as prey of grass snakes, as these studies tend to focus on adult snakes. These results would imply that competition may be at its greatest at a juvenile stage, when smooth snake diet is at its narrowest. These findings are in contrast to those of Luiselli and Rugiero (1991) who found that young grass snakes in Italy ate mainly amphibians with adults having a broader diet.

It is important to consider that, while grass snakes and smooth snakes are both colubrids, there may still be differences in digestion rates between them which may potentially affect and bias comparisons made between them, a potential problem not only for molecular analysis, but for conventional analysis of diet by visual examination too.

## 8.6 Conclusions

In the UK, where there is a narrow spectrum of reptile fauna and densities are low compared to continental Europe (Gasc *et al.* 1997), smooth snakes, which appear to be dependent on lizard prey as juveniles, are in decline and are restricted to areas of high lizard density. Pressure on them might be further exacerbated by competition with grass snakes at a juvenile stage, when both species are eating lizards. Management plans to maintain smooth

snake populations, relocate endangered colonies or attempts to restore their distribution to historical ranges, should focus on creating sufficiently high densities of lizards to sustain them by developing and maintaining optimum lizard habitats. This should include lizard surveys to identify hotspots where smooth snake reintroductions might be viable, with maintenance of lizard-friendly habitat and practices to support and increase preferential lizard prey. This work demonstrates for the first time that molecular techniques can provide a simple, quick and non-invasive means of studying reptile diet.

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## **Chapter 9**

### **Final Discussion**

## 9.1 *Molecular developments*

This study is the first to demonstrate that prey DNA is detectable and identifiable in the faeces of lizards and snakes.

When slow worm faecal DNA was amplified, cloned and sequenced with universal COI primers, all sequences ( $N=192$ ) belonged to the predator (results not presented), a common problem with their use (Jarman *et al.* 2004; Deagle *et al.* 2005). Therefore a method for biasing PCR in favour of prey was developed. Two potential methods were considered: the use of restriction enzymes and the use of a blocking oligonucleotide. Restriction enzymes were found to be ineffectual. When used prior to PCR they were not sufficient to adequately remove enough predator DNA to prevent its subsequent dominance in PCR; and when used after PCR they led to poor DNA yield and unsuccessful cloning attempts (transformation failure). The blocking probe, when designed to overlap with one of the universal primer sites, successfully prevented all predator amplification; but when designed to prevent primer elongation, failed to prevent predator domination. The method was independently developed by Vestheim and Jarman (2008) who also found that the method only worked when preventing primer annealing and not primer elongation. Faecal DNA from slow worms was amplified with universal primers in the presence of blocking probe at a concentration calculated to prevent amplification of up to 100ng/  $\mu\text{L}$  predator DNA and sent for pyrosequencing; however, the pyrosequencing reaction failed for unknown reasons (results not presented).

## 9.2 *Slow worm diet*

Slow worm diet has not been studied in depth before, yet land developers are legally required to translocate populations of them prior to development without knowledge of what constitutes a suitable habitat or how specialized their diet might be.

Pyrosequencing of slow worm faecal DNA with earthworm general primers revealed that their diet is comprised of numerous species and includes different functional groups of earthworms (epigeic, anecic, endogeic). The inclusion in their diet of deep-living species which only surface at night offered some insight into the unusual nocturnal foraging strategy of slow worms. The results also revealed that slow worms were not limited by a dependency on particular species / functional groups but were flexible in their choice of prey, with diet differing greatly between populations, presumably a reflection of different prey availability.

A more comprehensive analysis of 400 slow worms from different sites/habitats throughout the year, using a suite of pulmonates and earthworm primers, confirmed the importance of these prey in their diet throughout the year, and suggested they may have previously been underestimated. They confirmed the findings of Pedersen *et al.*'s (2009) study in Italy, that the diet of slow worms does not change ontogenetically in regards to predation on pulmonates and earthworms, most likely because these prey are present in all size classes. Also in agreement with Pedersen *et al.* (2009), it revealed that overall consumption of earthworms did not change seasonally. However, when considered at the level of individual prey species there were distinct and strong seasonal patterns of predation for each species. Seasonal patterns were also found for predation on pulmonates. As availability of these prey changes, slow worms may switch between them. Predation on *Arion* spp. Slugs revealed a sex bias, with female predation higher in the spring and the

autumn than male predation, which may have been the result of different nutritional needs driven by different reproductive costs.

These results provide a clearer picture of predation patterns by slow worms and demonstrate the benefit of molecular techniques over morphological identification which can seldom differentiate species or even families from digested remains.

Predation on many prey was influenced by weather (rainfall and temperature), known to affect surface activity of the prey. The results, therefore, indicate that availability of prey was driving predation on them in most cases rather than any preferential selection. In terms of pulmonates and earthworms, the major component of slow worms' diet, slow worms appear to be largely opportunistic.

### 9.3 *Parasitism of slow worms*

Various parasites have been recorded for slow worms in Eastern Europe, including nine species of parasitic nematode (Shimalov *et al.* 2000; Borkovcova and Kopriva 2005). Microscopy analysis of faeces of slow worms from the UK revealed just two parasites: *Neoxysomatium brevicaudatum*, a member of the Cosmocercoidae family; and a member of the Rhabditae family. PCR primers, developed to target the former species, were demonstrated to be as effective as establishing prevalence as time-consuming hand-sorting and microscopy. In a detailed analysis of prevalence in slow worms there was a male sex bias in the spring, which may be attributed to testosterone-induced immunosuppression. In the summer there was a higher female prevalence which may reflect seasonal variation in behaviour between males and females. In addition, the results indicate an initial increase in prevalence in younger animals followed by a decrease in prevalence in older animals. These

findings may be evidence that slow worms build up acquired immunity from exposure, but could equally be a result of older, more heavily infected animals, dying and being excluded from the study. Further work, including analysis of intensity (either obtained by faecal microscopy or by qPCR) would be useful, as would analysis of the *Rhabdita* nematode. Understanding the parasitic cycles of slow worms may be an important consideration in translocation programmes and management to ensure healthy populations.

#### 9.4 *Smooth snake and Grass snake diet*

The restricted distribution of smooth snakes in the UK is not fully understood but is frequently accepted as being a function of associated habitat loss, in particular, the decimation of heathland. However, as smooth snakes are not limited to heaths throughout Europe, and adders and grass snakes are far more wide-ranging in the UK, it is not a satisfactory explanation by itself. As prey have a strong influence on predator distribution (Fretwell and Lucas 1970; Fretwell 1972) a more in-depth analysis of the prey of smooth snakes was considered important to their conservation. Predation on a range of amphibians, reptiles, small mammals and invertebrates was analysed using species-specific primers.

The results confirmed that small mammals are an important component of smooth snake diet in the UK and that they are not solely reptile specialist as has been suggested (Duguy 1961; Bruno 1966; Andren and Nilson 1976, 1979; Street 1979; Drobenkov 1995; Rugiero *et al.* 1995). However, inclusion of mammals was found to increase in their diet with age, with juveniles and sub-adults apparently restricted to a reptile diet. The same trend was found for two different sites, although fewer small mammals were included in the diet

of smooth snakes at Wareham, an open habitat likely to support fewer mammals than the site at Ringwood.

Analysis of grass snake predation revealed much higher levels of reptile consumption than previously thought, with them found to consume lacertids (common lizards (*Lacerta vivipara*) and sand lizards (*L. agilis*)) in equal proportions to smooth snakes. Predation on small mammals was low, and predation on amphibians high, in accordance with the literature. These results suggest a greater level of potential competition between smooth snakes and grass snakes than previously thought. At a juvenile stage, when smooth snakes are restricted to a reptile diet, they are probably limited to areas with sufficiently high reptile densities.

These findings may have strong implications for their future conservation management and reintroduction programmes, as well as providing a more satisfactory explanation for their limited distribution.

Globally, reptiles are in decline from a number of threats, including habitat loss and degradation, introduced alien species, pollution, disease, parasitism and climate change (reviewed in Gibbons *et al.* 2000). There were 100 reptiles listed as “endangered” by The World Conservation Union (IUCN) in 2000, with a further 153 categorised as “vulnerable”, indicating that they are likely to become extinct if the trend continues. Now that we know that prey DNA can be detected in reptile faeces a whole new area of ecological study will be open to herpetologists for studying the trophic interactions of reptiles.

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