Molecular analysis of the diets of snakes: changes in prey exploitation during development of the rare smooth snake *Coronella austriaca*.

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Abstract

Reptiles are declining in many parts of the world, mainly due to habitat loss and environmental change. A major factor in this is availability of suitable food. For many animals, dietary requirements shift during developmental stages and a habitat will only be suitable for conserving a species if it supports all stages. 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 facilitate non-invasive analysis of diet from faeces without these constraints. We tested the hypothesis that diet changes during growth stages of smooth snakes (*Coronella austriaca*), which have a highly restricted distribution in the UK but are widespread in continental Europe. Small numbers of the sympatric grass snake (*Natrix natrix*) were analysed for comparison. Faecal samples were collected from snakes and prey DNA analysed using PCR, targeting amphibians, reptiles, mammals and invertebrates. Over 85% of smooth snakes were found to have eaten reptiles and 28% had eaten mammals. Predation on mammals increased with age and was entirely absent among juveniles and sub-adults. Predation on reptiles did not change ontogenetically. Smooth snakes may, therefore, be restricted to areas of sufficiently high reptile densities to support young snakes.

Introduction

The distributions of snakes in temperate regions may be strongly influenced by the presence of winter hibernation sites (Prior & Weatherhead 1996; Harvey & Weatherhead 2006) and by temperature and the ability to thermoregulate (Huey 1991; Reinert 1993; Row & Blouin-Demers 2006). However, the "ideal free distribution theory" (Fretwell & 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 & Qualls 2009), water pythons (*Liasis fuscus*) (Madsen & Shine 1996) and carpet pythons (*Morelia spilota metcalfei*) (Heard *et al.* 2004), for example, 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 particular stages of its life. Differences between juveniles and adults in their prey species selection, and the size of prey, have been observed in fish (McCormick 1998; Reñones *et al.* 2002), birds (Price & Grant 1984), mammals (Dickman 1988; Page *et al.* 2005) and reptiles (Herrel & O'Reilly 2006), and is commonly seen in snakes (Lind & Welsh 1994; Pizzatto *et al.* 2009; reviewed in Shine & Wall 2007). Frequently, juveniles eat smaller prey and a narrower range of species than adults. This may simply be a function of differences in relative body sizes of predators and prey, but can also be attributed to inexperienced foraging ability (Rutz *et al.* 2006), differential habitat use due to changes in predator avoidance / territory defense with age, or in order to reduce intraspecific competition (Angelici *et al.* 1997).

Reptiles in Britain, as elsewhere, are in decline (Wilkinson & Arnell 2011) as habitats are continually destroyed, fragmented or unsympathetically managed. Their ranges are increasingly becoming narrower, leading to extinctions in many regions (Prestt 1971; Howes 1973). In the UK, the smooth snake (*Coronella austriaca*) is considered endangered due to its severely restricted distribution to a few strongholds on heathlands 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 grass snake is found up to, and occasionally beyond, 56^{o}N, approximately the border of England and Scotland. Smooth snakes range almost as far north as grass snakes throughout mainland Europe, up as far as 60^{o}N in Sweden, which corresponds to a vegetational and climatological boundary (Gasc *et al.* 1997). Thus, a distribution in the UK that is restricted by temperature is unlikely. While smooth snakes are only found on sandy lowland heath in Britain, throughout continental Europe they are found in a variety of different habitats (pine forests, mixed riverside forests, bogs, vegetation bordering fields, bramble patches, orchards and open grassland (Beebee & Griffiths 2000)), and so habitat structure does not appear able to explain their UK distribution. Alternatively, 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; Andrén & Nilson 1976, 1979; Street 1979; Drobenkov 1995; Rugiero *et al.* 1995). However, their diet in the UK has been subject to debate, and while there is agreement over the main range of prey taken (amphibians, reptiles and small

mammals) the importance of each is unclear. Goddard (1981, 1984), using morphological analyses of faeces and regurgitates, found the proportion of smooth snakes which had consumed small mammals was more than twice that of smooth snakes that had consumed 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 revealed they were consuming lizards, snakes and mice in proportion to their ratios in the wild. However, juvenile smooth snakes have showed an innate feeding preference for lizards (Goddard 1984), suggesting 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 & Phelps 1977; 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 a very broad diet which includes amphibians, reptiles and birds, but predominantly small mammals (Prestt 1971; Drobenkov 1995), while grass snakes are thought to be amphibian specialists that take little other prey (Drobenkov 1995). Although there is 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 & Phelps 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 occasionally include reptiles in their diet (Luiselli & Rugiero

1991; Capula *et al.* 1994; Drobenkov 1995; Filippi *et al.* 1996; Luiselli & Capula 1997) and small mammals (Luiselli & Rugiero 1991; Luiselli & Capula 1997; Gregory & Isaac 2004) and smooth snakes have been found to eat amphibians (Nature Conservancy Council 1983), although these are considered to be a small components of their diets. However, snake size and age are seldom accounted for in these studies, which have usually been conducted on adults only and may be missing critical information if there are ontogenetic shifts in diets. If smooth snakes are dependent on a narrow range of specific prey as juveniles, 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.

Conventional analyses 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 vertebrates including fish (Saitoh *et al.* 2003; Jarman & Wilson 2004), birds (Jarman *et al.* 2004; Deagle *et al.* 2007), and mammals (Jarman *et al.* 2002, 2004; Marshall *et al.* 2010; Clare *et al.* 2009, 2011; Razgour *et al*. 2011). Next generation sequencing (NGS) has been successfully applied to analyse the diet of the legless lizard (*Anguis fragilis*) (Brown *et al.* 2012) and the effects of season and sex on the diet of the Turtle-headed sea snake (*Emydocephalus annulatus*) were also identified using a DNA sequencing approach (Goiran *et al.* 2013). Species-specific PCR primers, which are a less costly alternative to NGS, have not

previously been applied to analyses of reptile diet. Such molecular approaches allow standardized non-invasive screening of reptile faeces for target prey.

Here we used molecular tools to investigate predation by smooth snakes and address the hypothesis that there are ontogenetic changes in the diet of smooth snakes which may be responsible for their severely restricted distribution. In addition, a preliminary study was made on predation by sympatric grass snakes to investigate the potential for the approach to identify resource partitioning between these sympatric snakes.

Methods

Field sites and faecal collection

A total of 53 faecal samples were collected from smooth snakes during monthly visits to two English sites (Ringwood and Creech) from April–September in 2007 and 2008, the active period for British reptiles (Beebee & Griffiths 2000). The Ringwood site (50^{o}52'N, 1^{o}51'W) consists of just under a hectare of unimproved grassland adjacent to ericaceous heathland and coniferous woodland. The Creech site (50^{o}39'N, 2^{o}06'W) is an area of ericaceous heathland comprising common heather (*Calluna vulgaris*), bell heather (*Erica* cinerea) and gorse (*Ulex* spp.). Both sites are managed by The Herpetological Conservation Trust and are typical of habitats in Southern England where smooth snakes are found. The opportunity was also taken to collect further faecal samples from a small number of grass snakes (n=14), collected at the same time and from the same sites, to test the ability of the molecular detection methods on another species and to provide limited comparative information on their diets.

158 Faecal samples were collected into 2 mm microcentrifuge tubes by gentle palpatation
159 of the animals. Snout-vent length (SVL), used as a proxy for age, and total weight were
160 measured. All snakes were photographed, allowing individual identification based on unique
161 banding patterns and colouration. To avoid pseudoreplication, snakes previously caught
162 were excluded from analysis. The appropriate license was obtained from Natural England.
163
164 *DNA extraction, PCR and sequencing*
165 All animal material used for DNA extractions were donated by small mammal and
166 herpetological groups, having been found dead during animal surveys. Animals collected
167 included common vole (*Microtus arvalis*), field vole (*Microtus agrestis*), bank vole (*Myodes*
168 *glareolus*), common shrew (*Sorex araneus*), pygmy shrew (*S. minutus*), water shrew
169 (*Neomys fodiens*), brown rat (*Rattus norvegicus*), yellow necked mouse (*Apodemus*
170 *flavicollis*), house mouse (*Mus musculus*), palmate newt (*Lissotriton helveticus*), smooth
171 newt (*L. vulgaris*), common lizard (*Lacerta vivipara*), sand lizard (*L. agilis*), slow worm (the
172 legless lizard *Anguis fragilis*), common frog (*Rana temporaria*), adder (*V. berus*), grass
173 snake (*N. natrix*) and smooth snake (*C. austriaca*). The DNeasy® Tissue Kit (Qiagen) was
174 used for extraction of DNA from tissue. All DNA was amplified by PCR with the universal
175 forward primer LCO1498 (Folmer *et al*. 1994) and the reverse primer HCO1777 (5'-
176 ACTTATATTGTTTATACGAGGGAA-3') (Brown 2010) with the following conditions:
177 1X buffer, 2 mM $MgCl_2$, 0.5 mM dNTP (Invitrogen), 0.5 µM of each primer, 0.38 U *Taq*
178 polymerase (Invitrogen) and 2 µL of DNA in/ 25 µL PCR reaction with an initial
179 denaturation at 94 ^{o}C for 3 min, 35 cycles of 94 ^{o}C for 30 s, 48 ^{o}C for 30 s and 72 ^{o}C for 45
180 s, and a final extension at 72 ^{o}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 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^{o}C and 15 min at 80^{o}C. Cleaned product was then used in sequencing PCR using a Big Dye^{TM} terminator sequencing kit (Promega, Madison, WI, USA). Sequences were checked for errors using Sequencher 3.1.2.

DNA from faecal samples were extracted using the QIAamp® DNA Stool Mini Kit (Qiagen) in accordance with the manufacturer's instructions.

Species- and group-specific primer design

Cytochrome b sequences were downloaded from Genbank for the following species: smooth snake (Accession no. EU022673), water vole (*Arvicola amphibius*, AF159400), bank vole (EU035710), field vole (DQ663658), common shrew (GU827395), pygmy shrew (GU827394), yellow-necked mouse (AF159392), wood mouse (*Apodemus sylvaticus*, HQ158102), house mouse (AB125774), common frog (FJ030872), palmate newt (U55948), smooth newt (DQ821238) and red-spotted toad (*Bufo punctatus*, DQ085775, used as a proxy for *B. bufo*). Primers for common frog, smooth newt and small mammals were designed by eye using BioEdit (version 7.0.4.1) to align homologous sequences and NetPrimer (Premier Biosoft International) to check for self-dimers, cross-dimers, hairpin structures and melting temperatures. Cytochrome oxidase I sequences were downloaded from Genbank for smooth

snake (AY122752) and grass snake (AY122664) and aligned with sequences for slow worm, common lizard and adder. Primers were designed for slow worm and common lizard.

Other primers used included those for bank vole (BV-CG95 and BV-CG266), common shrew (SA520 and SA628) and pygmy shrew (SM421 and SM544), targeting cytochrome b (Moran *et al.* 2008), plus group-specific primers for earthworms (185F and 14233R) (Harper *et al.* 2005) and arionid slugs (Harper *et al.* 2005), which target the 12S rRNA region. Species-specific primers were designed or selected for prey species known to be common components of smooth snake and grass snake diet (Drobenkov 1995).

Primer optimization and screening

A temperature gradient PCR was performed for each primer set to determine the highest temperature at which the target DNA would amplify. Each primer pair was tested for target-specificity against DNA from all other potential prey species. PCR was performed using a Peltier Thermal Cycler (Bio-Rad Laboratories, CA, USA). PCR concentrations used were the same as those described above, but with a PCR cycle of 94 ^{o}C for 3 min, 35 cycles of 94 ^{o}C for 30 s, the highest working annealing temperature for that primer pair for 45 s and 68 ^{o}C for 45 s, and a final extension at 68 ^{o}C for 10 min.

Specificity was achieved for common shrew, common frog, smooth newt, common lizard, slow worm and the small mammals (Table 1). The bank vole primers CG95/CG266 (Moran *et al.* 2008) cross-amplified with field vole at all temperatures, but with no other taxa at 58 ^{o}C. The pygmy shrew primers SM421/SM544 (Moran *et al.* 2008) cross-amplified with common shrew and water shrew at all temperatures, but were group-specific to all shrews at 53 ^{o}C. Between 52 ^{o}C and 64 ^{o}C the common shrew primers SA520/SA628 (Moran *et al.*

2008) resulted serendipitously in bands that were species-specific in pygmy shrews (with a *ca.*150 base pair fragment) and water shrew (with a *ca.*250 base pair fragment), both distinguishable from the *ca.*200 bp fragment for common shrew. These may be the result of amplification of pseudogenes, but they proved to be reliable species-specific markers that could separate the three species of shrew in snake faeces. The common lizard primers LCO1498/LV1714R cross-amplified with sand lizard between 53-62 ^{o}C and were used as general lacertid primers at 53 ^{o}C.

All faecal samples were screened with each primer pair twice. Target DNA was included as a positive control, to ensure PCR success, and water was included as a negative control to check for contamination.

Statistics

The effects of smooth snake SVL, weight and sex, along with site, month, year, temperature, rainfall and sunshine on predation of various prey were explored within a Generalised Linear Model (GLM). Weight, SVL, temperature, rainfall and sunshine were treated as covariates and all other predictors as factors. Weather information was obtained from the Met Office. The effects of grass snake SVL, only, were considered within GLMs investigating their predation on prey, due to the small sample size. A binomial error distribution was used with a logit link function. All analyses were conducted in the R version 2.8.2. Patterns of predation by the two snake species on each prey species were analysed. However, comparisons between prey were not made due to possible differences between primers in the ability of their amplicons to survive digestion (King *et al*. 2008).

Results

Predation by smooth snakes

The primary prey of smooth snakes was reptiles (Fig. 1), with no significant effect of predator age/SVL on their consumption. However, there was a significant effect of both snake SVL and site on predation of shrews, with the probability of predation increasing with snake size ($\chi^2 = 10.4$, df = 1, P=0.003, Fig. 2a) and a much higher probability of predation at Ringwood (n=24) than at Wareham (n=29) ($\chi^2 = 8.8$, df = 1, P=0.001, Fig. 2a). Similar effects of SVL and site were also seen when predation on all small mammals combined was analysed (SVL: $\chi^2 = 5.5$, df = 1, P=0.020; site: $\chi^2 = 5.0$, df = 1, P=0.026, Fig. 2b).

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). Predation on reptiles fluctuated between months but was high throughout the entire season. Even in August, when predation on reptiles was at its lowest, it was still above 50%. Predation on reptiles between the two sites did not significantly differ, with 85.7% of smooth snakes at Ringwood and 83.3% at Wareham having consumed them.

Predation on earthworms (18%) and slugs (0%) was minimal or absent and there was no significant effect of any of the variables considered. Predation on smooth newts (3%) and common frogs (9%) was too low to explore statistically.

Predation by grass snakes

Prey detection in grass snakes was also successful, although results should be treated with caution given the small sample size (N=14). Snake SVL had a highly significant negative effect on predation on reptiles (SVL: $\chi^2 = 10.4$, df = 1, P=0.001), with all grass snakes below 550mm in SVL (*n=10*) testing positive for reptile DNA but all those above 600mm (*n*=4) testing negative.

There was no effect of grass snake SVL on newt predation. All other prey (small mammals, common frog and earthworm) were preyed on too infrequently for statistical analysis.

Comparison of smooth snake and grass snake diet

Predation on small mammals by smooth snakes was 28%, 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. 1). There was no significant difference in predation by the two snake species on common lizards or 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 higher in grass snakes than in smooth snakes (Fisher's exact test, P<0.001).

Discussion

Smooth snakes

The focus of this study was on the diet of smooth snakes, reflecting interest in the conservation of this species and its unusual and restricted distribution patterns. The main prey of these snakes (N=53) was found to be other reptiles (84.5% tested positive) followed by small mammals (28.0%).

Predation on reptiles was similar at each of the sites, with 85.7% of smooth snakes at Ringwood and 83.3% at Wareham having consumed them. However, predation on small mammals differed between the two sites, with twice as many testing positive at Ringwood (38.3%) as at Wareham (16.7%), probably reflecting differences in prey availability at the two locations. 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. These results indicate that small mammals may not be 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.

Smooth snakes showed increased predation on shrews (P=0.003) and small mammals generally (P=0.020) as they grew larger. Taking SVL as a proxy for age (Bronikowski & Arnold 1999; Gignac & Gregory 2005), this indicates an ontogenetic shift in smooth snake diet, with very few small mammals taken when the snakes are young but increasing predation as they grow. This may be explained either by a greater initial preference for reptile prey or by an inability to find, handle or consume small mammals when young (Shine & Wall 2007). No smooth snakes below 300 mm in SVL, equating approximately to a three year old snake (Goddard 1984), were found to have consumed any

small mammals, so in these first few years their diet was likely to have been almost exclusively reptile. There was no change in predation on reptiles (common lizard, lacertids generally or slow worm) with snake size, with predation on them starting when smooth snakes were as small as 190 mm in SVL, within their first year. Most probably the youngest smooth snakes are eating juvenile lizards. Thus they continue eating lizards throughout their life, while incorporating small mammals as they grow larger / older.

If the geographical distribution of smooth snakes in the UK is restricted by prey availability then it is most likely that this restriction is at the juvenile stage, when their diet is at its narrowest and they are almost entirely dependent on juvenile lizards. While smooth snakes are clearly capable of eating invertebrate prey, only 17% were found to have consumed earthworms, and juveniles were no more likely to consume them than adults. No snakes were found to have consumed any *Arion* slugs despite their abundance at the field sites. It is quite possible that positives recorded for earthworm consumption by smooth snakes were in fact the result of secondary predation (Harwood *et al.* 2001; Sheppard *et al.* 2005). Slow worms were shown to be major consumers of earthworms in a separate study (Brown *et al.* 2012) and therefore earthworm DNA may have ended up in the guts of smooth snakes following slow worm consumption. Based on tongue-flick experiments, Pernetta *et al.* (2009) found that smooth snakes showed a preference for the scent of lizard and mammal prey over invertebrates, even as juveniles. Van de 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

341 determined by the distribution of lizard prey. However, it may be that smooth snakes are
342 restricted not just to areas where lizards are present, but to areas with a sufficiently high
343 density of juvenile lizards. The heaths of southern England have higher densities of common
344 lizards, sand lizards and slow worms than anywhere else in the country (Braithwaite *et al.*
345 1989).
346
347 *Grass snakes*
348 Grass snakes are usually associated with damp and aquatic environments, hunting the prey
349 found in these habitats, particularly amphibians (Drobenkov 1995; Gregory & Isaac 2004).
350 Although sample size was limited, it was also apparent that amphibians were a major dietary
351 component, with 64.3% testing positive (mainly for smooth newts) compared with a rate of
352 just 5.2% in smooth snakes. Predation by grass snakes on small mammals was exclusively
353 on water shrews, again an aquatic prey. Interestingly, however, a larger proportion of grass
354 snakes were found to be consuming reptile prey (68.2%, Fig. 1) than previous studies have
355 found (Drobenkov 1995; Gregory & Isaac 2004). There was no significant difference
356 between consumption of common lizards by grass snakes and smooth snakes, indicating the
357 potential for competition between these species.
358
359 *Analysis by PCR*
360 Molecular diagnostics revealed detailed and clear information on reptile diets and the effects
361 of developmental stage on prey choice. This approach allows for standardized non-invasive
362 analyses and monitoring of diets, particularly cost- and time-effective where prey-specific
363 primers are already developed. There are potential limitations to these approaches: prey

364 species may be digested at different rates which may affect detectability (e.g. Deagle &
365 Tollit 2007), and primers may differ in sensitivity (Symondson 2002), but these potential
366 biases can be reduced by targeting DNA amplicons of a similar size and on the same gene or
367 by evaluating sensitivity by serial dilution tests (e.g. Chen *et al.* 2000). Unlike some
368 traditional methods, such as forced regurgitation, it is not possible to determine the size of
369 prey or the number of prey individuals consumed by a predator and where this information is
370 desired a combination of approaches is the best possible practice.

371 In this study, with a sample of just 14 grass snakes taken opportunistically, it is too
372 early to project any conclusions onto the wider population, although these findings
373 corroborated many previous studies of grass snake diet (Drobenkov 1995; Gregory & Isaac
374 2004) while also hinting that predation on slow worms may be higher than thought at sites
375 such as these where they are abundant.

376 UK smooth snakes were shown to be almost entirely dependent on lizard prey as
377 juveniles, restricting them to areas of high lizard density. Management plans to maintain
378 smooth snake populations, relocate endangered colonies or attempts to restore their
379 distribution to historical ranges, should focus on creating optimum lizard habitats. This
380 should include lizard surveys to identify hotspots where smooth snake reintroductions might
381 be viable, with maintenance of lizard-friendly habitat. This study offers both insight into the
382 limited distribution of smooth snakes and presents a new tool to aid reptile conservation.

383

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566

Table 1. Species- and group-specific primers, with target mitochondrial gene, optimised annealing temperature and amplified product size.

	PRIMERS				
TARGET SPECIES/GROUP	**FORWARD**	**REVERSE**	**GENE**	**ANNEALING TEMP. (°C)**	**PRODUCT SIZE**
Common frog	RTF (TACAGCCGATACCTCCCTC)	RTR (TTCATGTCTCTTTGTAGAGG)	cytb	62	176
Smooth newt	LHF (GACTCGTACGAAACATCCA)	LHR (CGCCTATATATGGAATAGCGG)	cytb	55.5	243
Common lizard	LCO1498 (Folmer *et al.* 1994)	LV1714R (CCCGAACCCACCAATTATTAC)	COI	62	216
Lacertid spp.	LCO1498 (Folmer *et al.* 1994)	LV1714R (CCCGAACCCACCAATTATTAC)	COI	53	216
Slow worm	LCO1498 (Folmer *et al.* 1994)	AF1608R GGCTGGCTTAACTCTGCG	COI	54	110
Small mammal spp.	MM14701 (TGACAAACATACGAAAAACACACCCAT)	MM14905 (ATGTGTGTTACTGATGAAAAGGCTGTTAT)	cytb	55.5	206
Bank / field vole	CG95 (Moran *et al.* 2008)	CG266 (Moran *et al.* 2008)	cytb	58	171
Common shrew	SA520 (Moran *et al.* 2008)	SA628 (Moran *et al.* 2008)	cytb	64	108
Pygmy shrew	SA520 (Moran *et al.* 2008)	SA628 (Moran *et al.* 2008)	cytb	52	*ca.*150
Water shrew	SA520 (Moran *et al.* 2008)	SA628 (Moran *et al.* 2008)	cytb	52	*ca.*250
General shrew spp.	SM421 (Moran *et al.* 2008)	SM544 (Moran *et al.* 2008)	cytb	53	108
Earthworm spp.	185F (Harper *et al.* 2005)	14233R (Harper *et al.* 2005)	12S	65	225-236
Arion spp.	Ai1F (Harper *et al.* 2005)	AR2R (Harper *et al.* 2005)	12S	57	208-221

Figure legends

Figure 1. 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.

Figure 2. Predicted probability of predation by smooth snakes (with SE, dotted line) on **a)** shrews (common and pygmy) and **b)** all small mammals, showing significant difference between sites and a significant effect of snake length (determined by GLM).

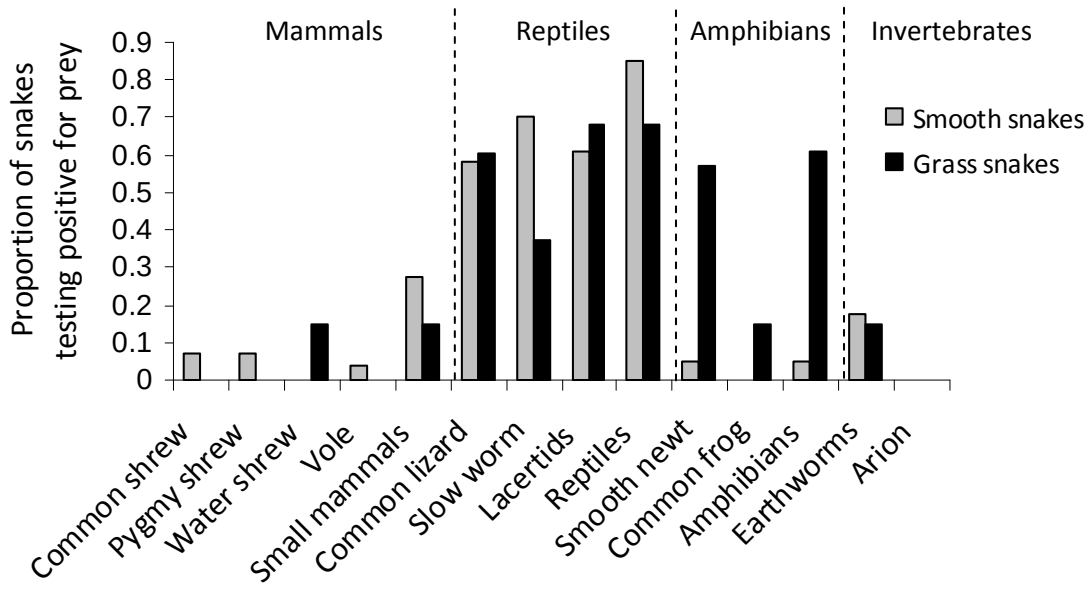

Mammals
Reptiles
Amphibians
Invertebrates
Proportion of snakes testing positive for prey
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Smooth snakes
Grass snakes
Common shrew
Pygmy shrew
Water shrew
Vole
Small mammals
Common lizard
Slow worm
Lacertids
Reptiles
Smooth newt
Common frog
Amphibians
Earthworms
Arion

Figure 1.

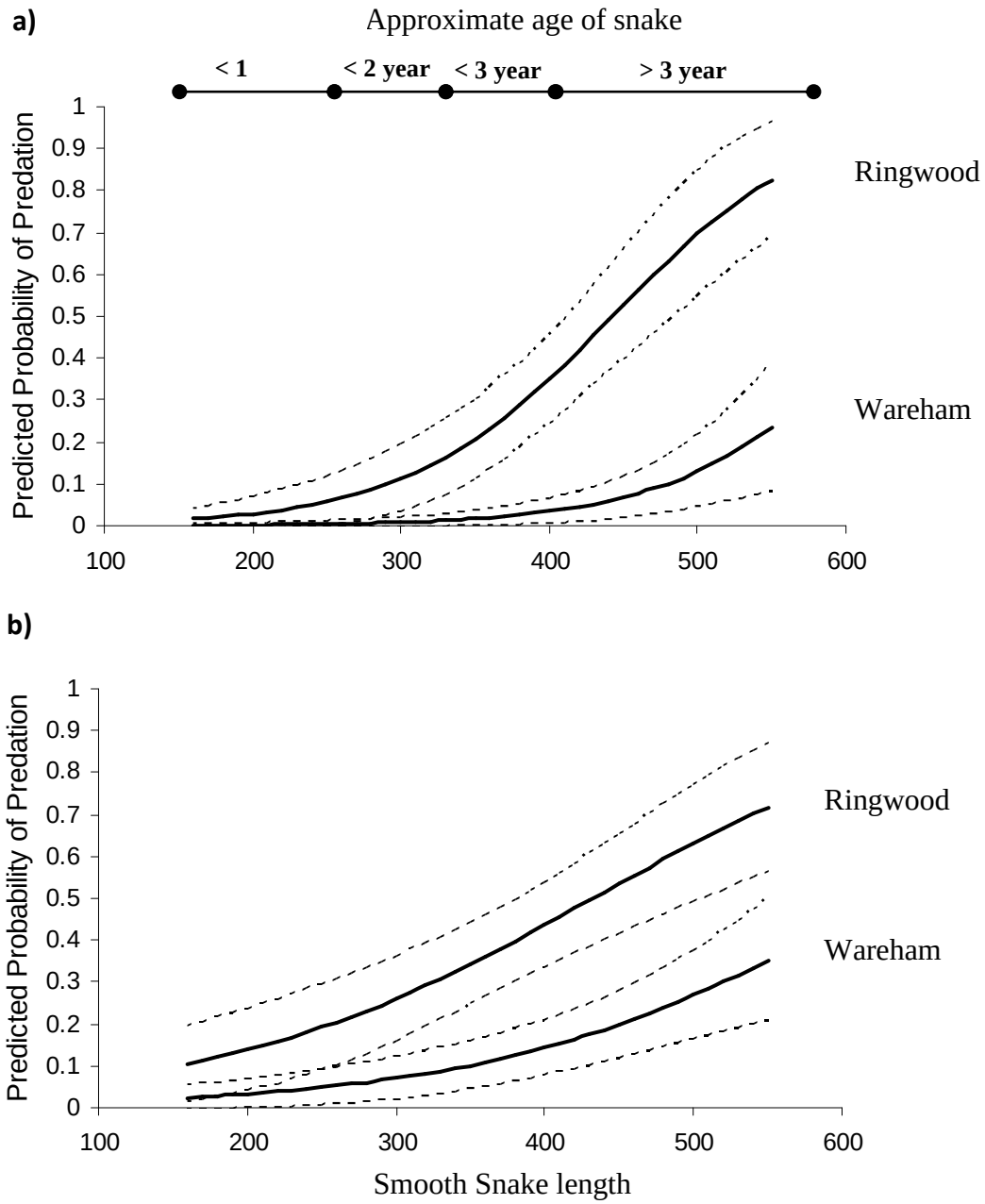

a)
Approximate age of snake
< 1
< 2 year
< 3 year
> 3 year
Predicted Probability of Predation
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
100
200
300
400
500
600
Ringwood
Wareham
b)
Predicted Probability of Predation
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
100
200
300
400
500
600
Smooth Snake length
Ringwood
Wareham

Figure 2.

Year	Month	Sex	Site	SVL.cm	VTL.cm	Total.Lengt	Weight	Mean.Tem	Rainfall.mn
2007	August	Female	Wareham	190	40	230	9	19.4	77
2007	August	Male	Ringwood	145	130	275	20	19.4	77
2007	August	Female	Wareham	250	50	300	20	19.4	77
2007	August	Male	Wareham	250	60	310	20	19.4	77
2007	August	Female	Ringwood	355	75	430	30	19.4	77
2008	August	Female	Ringwood	280	85	365	11.5	18.6	35.1
2008	August	Male	Wareham	360	100	460	36.7	20.2	92.8
2008	August	Male	Wareham	360	100	460	25	20.2	92.8
2008	August	Male	Wareham	420	130	550	39.5	20.2	92.8
2007	July	Female	Ringwood	180	40	220	10	19.8	121.7
2007	July	Female	Wareham	310	70	380	21	19.8	121.7
2007	July	Female	Ringwood	370	80	450	34	19.8	121.7
2007	July	Male	Wareham	350	115	465	35	19.8	121.7
2007	July	Male	Wareham	365	115	480	42	19.8	121.7
2007	July	Male	Wareham	365	115	480	42	19.8	121.7
2008	July	Male	Ringwood	160	30	190	4	20.2	92.8
2008	July	Female	Wareham	330	60	390	26	19.3	158.3
2008	July	Male	Ringwood	390	80	470	29	19.3	158.3
2008	July	Male	Ringwood	440	100	540	33	19.3	158.3
2007	June	Female	Ringwood					19.6	123.8
2007	June	Female	Ringwood					19.6	123.8
2007	June	Female	Wareham					19.6	123.8
2007	June	Female	Wareham	340	60	400	33	19.6	123.8
2007	June	Male	Ringwood	345	100	445	42	19.6	123.8
2007	June	Male	Ringwood	345	110	455	37	19.6	123.8
2008	June	Male	Wareham	250	60	310	10	19.1	44.4
2008	June	Female	Ringwood	260	60	320	15	19.1	44.4
2008	June	Male	Wareham	310	80	390	16.8	19.1	44.4
2008	June	Male	Wareham	330	70	400		19.1	44.4
2008	June	Male	Ringwood	340	110	450	36	19.1	44.4
2008	June	Male	Wareham	380	100	480	37.7	19.1	44.4
2008	June	Male	Wareham	380	115	495	32	19.1	44.4
2008	June	Male	Wareham	400	120	520	48.9	19.1	44.4
2007	May	Female	Wareham					19.6	123.8
2007	May	Male	Ringwood	210	50	260	10	19.6	123.8
2007	May	Male	Wareham	255	65	320	18	16.6	119.4
2007	May	Female	Wareham	340	60	400	33	16.6	119.4
2007	May	Female	Ringwood	340	75	415	23	16.6	119.4
2007	May	Male	Ringwood	320	100	420	55	16.6	119.4
2007	May	Female	Ringwood	360	60	420	40	16.6	119.4
2008	May	Male	Ringwood	250	50	300	11.4	18.3	79.8
2008	May	Male	Ringwood	250	60	310	14.2	18.3	79.8
2008	May	Male	Ringwood	290	90	380	16.7	18.3	79.8
2008	May	Female	Ringwood	380	60	440	30	18.3	79.8
2008	May	Male	Ringwood	370	90	460	33	18.3	79.8
2007	September	Female	Ringwood					18.6	35.1
2007	September	Female	Wareham					18.6	35.1
2007	September	Male	Ringwood	260	80	340	12.4	18.6	35.1
2007	September	Male	Ringwood	350	80	430	24.3	18.6	35.1
2007	September	Male	Ringwood	370	100	470	31.1	18.6	35.1
2008	September	Male	Ringwood	240	60	300	6.4	17.7	82
2008	September	Female	Wareham	350	80	430	14	17.7	82
2008	September	Male	Wareham	420	130	550	27.7	17.7	82

Supplementary Material S2. Forward and reverse *cytochrome b* primers designed for a) common frog, b) smooth newt and c) small mammals showing alignments with other British amphibian, reptile and small mammal species. Reverse COI primers designed for d) common lizard and e) slow worm showing alignments with other British reptile species. LCO1498 (Folmer *et al.* 1994) was used as the forward primer with each COI reverse. (~) given where no sequence data was available.

	Prey species	*Forward primer*	*Reverse primer*
a)	**Common frog**	**5′-**CCTCTACAAAGAGACATGAA-3′	5′-TACAGCCGATACCTCCCTC-3′
	Smooth newt	CATATTTAAAGAGACCTGAA	TACAGCAGACACACAATCA
	Palmate newt	CATATTTAAAGAGACATGAA	CACAGCAGACACACAATCA
	Red-spotted toad	TCTCTTTAAAGAGACCTGAA	CACAGCTGATACATCCATA
	Smooth snake	CCTAAATAAAAACGTCTGAC	CACAGCTAACATTAACCTT
	Water vole	CACCTTCATAGAAACATGAA	TACATCAGACACAATAACA
	Bank vole	CAATATAATTGAAACCTGAA	TACATCAGACACATCAACA
	Field vole	CAACATAATCGAAACATGAA	TACATCAGACACAGCAACA
	Common shrew	CATATACTTAGAAACATGAA	CACATCAGACACAATAACT
	Pygmy shrew	TATATACTTAGAAACATGAA	CACATCAGACACAATAACT
	Yellow-necked mouse	CAACATAATTGAAACCTGAA	CACATCAGATACATCAACA
	Wood mouse	TATTTTTATAGAAACATGAA	CACATCAGACACAATAACA
	House mouse	TACATTTATAGAAACCTGAA	CACATCAGATACAATAACA

	Prey species	*Forward primer*	*Reverse primer*
b)	**Smooth newt**	5′-GATTAGTGCGAAACATTCA-3′	5′-CGCCTATATATGGGATCGCTG-3′

Common frog	GACTCCTTCGTAATCTTCA	AGCCAATGTAGGGGGCGGCTG
Palmate newt	GACTCGTACGAAACATCCA	CGCCTATATATGGAATAGCGG
Red-spotted toad	GACTCCTACGCAACCTCCA	TTCCAATATATGGAGCAGCGG
Smooth snake	GAATAATACAAAACCTACA	~~~~~~~~~~~~~~~~~~~~
Water vole	GATTAATTCGATATTTACA	TTCCGATGTATGGAATTGCTG
Bank vole	GACTTATTCGCTATATACA	TGCCGATGTAAGGGATAGCTG
Field vole	GACTTATCCGATATATACA	TGCCTACGTAGGGGATGGCTG
Common shrew	GACTAATCCGATACCTTCA	AGCCGATATAAGGGATTGCTG
Pygmy shrew	GACTAATCCGCTATCTCCA	AGCCGATGTAAGGGATTGCTG
Yellow-necked mouse	GGCTGATCCGCTATACCCA	TGCCGATGTAGGGGATGGCTG
Wood mouse	GACTAATTCGATATATACA	TTCCGATGTATGGAATTGCTG
House mouse	GACTAATCCGATATATACA	TTCCAATATATGGGATGGCTG

Prey species	***Forward primer***	***Reverse primer***
c) Small mammals	5′-TGACAAACATACGAAAAACACACCCAT-3′	5′-ATATGGGCGATAGATGAGAATGCGAGGGA-3′
Common frog	~~~~~~~~~~~~~~~~~~~~~~~~~~~~	ATGTGAGCAACTGACGAGAATGCTGATTG
Smooth newt	CCCACACTTTACGAAAGACCCATCCCT	ATGTGGGCTACTGATGAGAATGCTGATTG
Palmate newt	CCCACCCTATACGAAAAACCCATCCGC	ATGTGGGCTACAGATGAGAAAGCTATGGA
Red-spotted toad	~~~~~~~~~~~~~~~~~~~~~~~~~~~~	ATATGAACAACGGATGAGAAGGCAAGGTT
Smooth snake	~~~~~~~~~~~~~~~~~~~~~~~~~~~~	ATATGAGTTACTGAAGAGAATGCTGTTAT
Water vole	TGACAAACATTCGAAAAACACACCCCC	ATGTGGGCAACTGATGAGAATGCTGTTGA
Bank vole	~~~~~~~~~~~~~~~~~~~~~~~~~~~~	ATGTGGGCTACTGATGAGAATGCTGTTGC
Field vole	~~~~~~~~~~~~~~~~~~~~~~~~~~~~	ATGTGTGTGACTGATGAGAAAGCAGTTAT
Common shrew	~~~~~~~~~~~~~~~~~~~~~~~~~~~~	ATGTGCGTGACTGATGAGAAGGCAGTTAT
Pygmy shrew	~~~~~~~~~~~~~~~~~~~~~~~~~~~~	ATATGGGCGACTGATGAAAATGCTGTTGA
Yellow-necked mouse	TGACAATTATTCGAAAAAAACATCCAT	ATATGGGTCACTGAAGAAAATGCTGTTAT

Wood mouse	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	ATGTGTGTTACTGATGAAAAGGCTGTTAT
House mouse	TGACAAACATACGAAAAACACACCCAT	~~~~~~~~~~~~~~~~~~~~~~~~~~GAGGGA

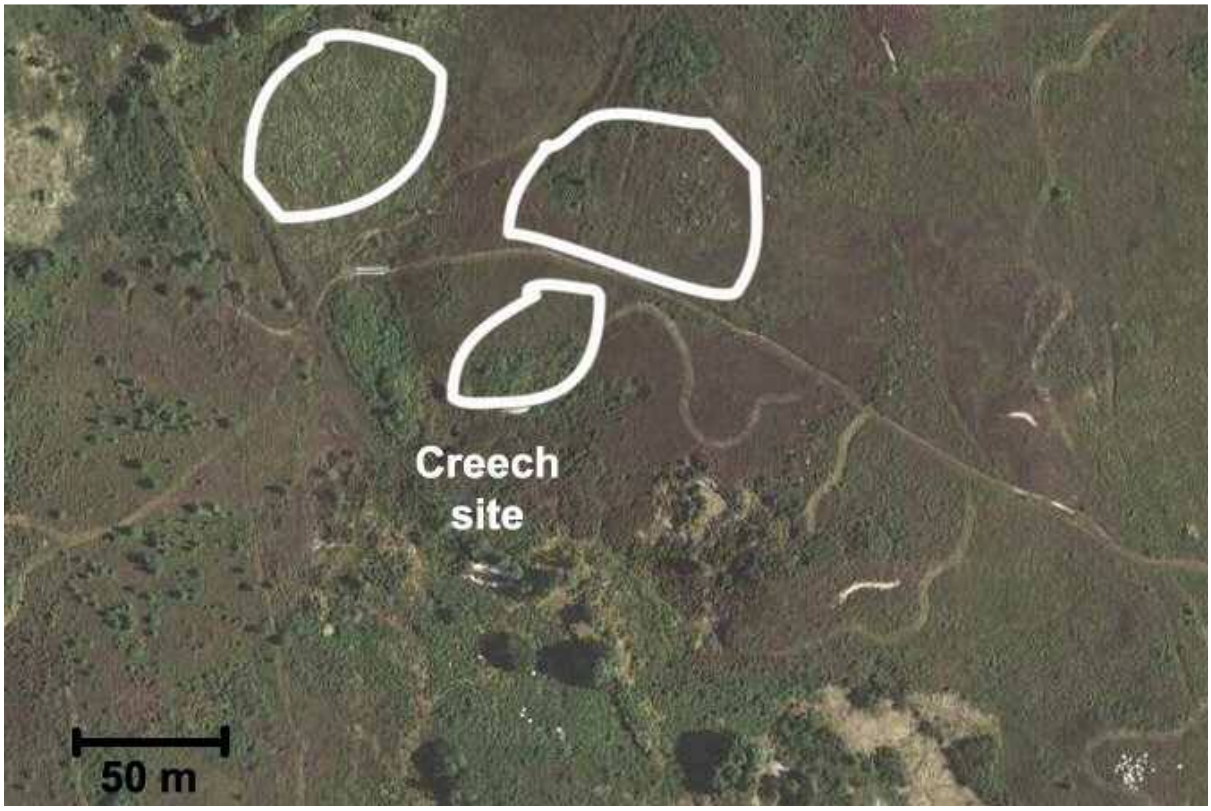
	Prey species	***Reverse primer***
d)	**Common lizard**	5′-CCCGAACCCACCAATTATTAC-3′
	Slow worm	~CCGAATCCGCCGATCATAAT
	Smooth snake	ATGTATCAACATAAAACCTAA
	Grass snake	GTGTATTAATATAAAACCTAA
	Adder	~CCAAAGCCCCCGATTATAAT

	Prey species	***Reverse primer***
e)	**Slow worm**	5′-GGCTGGCTTAACTCTGCG-3′
	Common lizard	GGTTGGCTTAGTTCGGTT
	Smooth snake	GCAGCAGCAATTACCATA
	Grass snake	GCGGCAGCGATTACTATA
	Adder	GGCTGAGTGAGTTCTATT

Table S3

	Number of predators testing positive for prey			
	Smooth snakes (*N=53*)		Grass snakes (*N=14*)	
	n	**%**	***n***	**%**
Pygmy shrew	3	5.7	0	0.0
Water shrew	0	0.0	2	14.3
Bank vole	2	3.8	0	0.0
Small mammals	15	28.3	2	14.3
Common lizard	31	58.5	9	64.3
Slow worm	38	71.7	5	35.7
Lacertids	33	62.3	5	35.7
Reptiles	45	84.9	10	71.4
Smooth newt	2	3.8	8	57.1
Common frog	0	0.0	2	14.3
Amphibians	2	3.8	9	64.3
Earthworms	9	17.0	2	14.3
Slugs (*Arion* spp.)	0	0.0	0	0.0


Ringwood
site
50 m


Creech
site
50 m

S2.

a)

b)