DOCTOR OF PHILOSOPHY

The ecology, biogeography, and taxonomy of isolated snake populations

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Award date:
2024

Awarding institution:
Bangor University

PRIFYSGOL
BANGOR
UNIVERSITY

The ecology, biogeography, and taxonomy of isolated snake populations

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In candidature for the degree of

Doctor of Philosophy

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Knowledge Economy Skills Scholarships (KESS 2) is a pan-Wales higher level skills initiative led by Bangor University on behalf of the HE sector in Wales. It is part funded by the Welsh Government's European Social Fund (ESF) convergence programme for West Wales and the Valleys.

This research was funded by the KESS 2 Programme and conducted in collaboration with the Welsh Mountain Zoo in Colwyn Bay, North Wales.

Ysgoloriaethau Sgiliau Economi Gwybodaeth
Knowledge Economy Skills Scholarships

UNDEB EWROPEAIDD
EUROPEAN UNION

Llywodraeth Cymru
Welsh Government

Cronfa Gymdeithasol Ewrop
European Social Fund

Molecular Ecology and Evolution @ Bangor
Prifysgol Bangor University

WELSH MOUNTAIN ZOO COLWYN BAY

Acknowledgements

Firstly, I extend my thanks to my lead supervisor Wolfgang. Throughout the PhD your enthusiasm, guidance, and support has been second to none. You have taught me an immense amount and equipped me with the tools to be an independent scientist, for which I am very grateful. Thank you also to Axel Barlow for all the genomics guidance, and training, and to Johanna Paijmans for invaluable help in assembling the genome and beyond. I am grateful to Michael Pook for generously hosting me, and for a great time in Nottingham. Thanks too to John Mulley for your invaluable help, and John Latchford for helping navigate the bureaucracy of Home Office licencing. Thank you also to Matt Hayward for supporting me remotely from Australia, and Ben Marshall for stats advice, for reassuring me of the quality of my code, and being a great friend and sounding board. Thanks also to Professor Richard Griffiths for stats training in Kent.

A massive thank you to the staff of the Welsh Mountain Zoo. So many people to thank for their tireless help with the project. Nick Jackson for taking on the project - without your fascination for the snakes it would never have happened. Thanks also to Kim Wood who assisted with equal enthusiasm in Nick's stead, and who like Nick contributed as a snake finder during the study. Similarly, Peter Litherland, for helping everything to go smoothly, catching snakes for us, and bending over backwards to make sure we had everything we needed – thank you. Thanks also to Amy, Tom L, Tom W, Jo, Claire, Simon, Kieran, Steve, Becky, Di, Margaret, Dave, Niall, Harry, Andy, Will, Tracy, Charlotte, and the many others who found snakes, passed on tips, or just passed the time of day when we were snake tracking. It was a privilege.

To my superb field team: assistants Devlan Alkins, Guillem Limia Russel, Rebecca Bracegirdle, Antonio Gandini, Andrea Pozzi, and tireless master's student Lauren Jeffrey and her team Henry Crisp and Jack Pearce. Thank you for making fieldwork great through your enthusiasm and dedication, and the fact you are all fun to be around. To Rhys Morgan – thank you for being a steadfast teammate during transmitter implantations. Also thank you to Ben Owens and Ellie Morris for guiding me in the lab, and all the other MEEB students and staff for fostering such a collaborative environment.

To the people of Colwyn Bay who allowed us at various times to rummage around in their compost heaps and come into their gardens in pursuit of snakes – thank you. Especially Mary, John and Alison, Wynn and Stephanie, John and Stephanie, and John, for their incredible generosity. There

are others who helped a great deal who do not want their snake locations telegraphed but thank you too. Also, many thanks to the staff of London Zoo who provided samples for the genetic analysis. I must also thank the KESS II programme staff, especially Penny Dowdney, Dawn Davies, and Brian Murcutt, who swiftly offered help whenever required and ensured smooth running despite difficult circumstances during the pandemic.

To my Mum and Dad for their unwavering encouragement and support, and for fostering my enthusiasm for reptiles – aided by my grandparents – thank you. Thanks also to my brother Will for being a supportive ally throughout. Many thanks to Kate Taylor and Ravi Gill for generously hosting me during my write up and for plying me with crucial amaretto sours. Lastly, I would like to thank my partner Maya Gill-Taylor for encouraging me to pursue even more education, for moving to Wales with me so I could, and for always being there.

Declaration and consent

I hereby declare that this thesis is the results of my own investigations, except where otherwise stated. All other sources are acknowledged by bibliographic references. This work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree unless, as agreed by the University, for approved dual awards.

I confirm that I am submitting this work with the agreement of my Supervisor(s).

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‘Yr wyf drwy hyn yn datgan mai canlyniad fy ymchwil fy hun yw’r thesis hwn, ac eithrio lle nodir yn wahanol. Caiff ffynonellau eraill eu cydnabod gan droednodiadau yn rhoi cyfeiriadau eglur. Nid yw sylwedd y gwaith hwn wedi cael ei dderbyn o’r blaen ar gyfer unrhyw radd, ac nid yw’n cael ei gyflwyno ar yr un pryd mewn ymgeisiaeth am unrhyw radd oni bai ei fod, fel y cytunwyd gan y Brifysgol, am gymwysterau deuol cymeradwy.

Rwy'n cadarnhau fy mod yn cyflwyno'r gwaith hwn gyda chytundeb fy Ngoruchwyliwr (Goruchwylwyr).

Abstract

Throughout time, shifts in species ranges caused by changes in climate and environment have created isolated populations of animals, presenting them with a suite of genetic and behavioural challenges. In modern times, human-mediated transport of novel species and the fragmentation of habitats lead to the creation of numerous isolated populations. Here, we use the Aesculapian snake (*Zamenis longissimus*) as a model species which has a broad natural distribution and multiple introduced populations. We investigate the behaviour of this snake in its introduced range in North Wales using radiotelemetry of wild snakes. Our results suggest a reliance on human features of their semi-rural landscape. Using whole-genome sequencing, we compare the genetic structure and health of multiple native populations of Aesculapian snakes with the introduced populations in Wales and England. Then, we infer the biogeographic history of Aesculapian snake populations. We find the population in Wales to be of uncertain, possibly mixed ancestry, and the population in London to have stemmed from an introduction of Italian specimens. Finally, we examine a long-isolated population natural population of rinkhals, a venomous snake from Zimbabwe. Using museum DNA approaches, we demonstrate evidence of speciation resulting from this isolation, and formally describe the Nyanga rinkhals (*Hemachatus nyangensis*).

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Chapter 1: General introduction

1.1 Introduced species

Animal ranges have changed dramatically through time because of shifts in climate, topography, and resulting vegetational structure. In the Anthropocene, human beings have become the major agent of change influencing the distribution of species (Bergman et al., 2023), and the globalised carriage of freight and goods has the power to introduce entire new populations of animals to distant places (Hulme, 2009). These shifts in the ranges of animals have occurred on such a scale, and for so long, that it is no longer possible to identify the natural distribution of some species (Khedkar et al., 2022). As human-mediated introductions show no sign of abating, the determinants of success in colonising new environments remains a central theme in evolutionary biology and ecology (Chapple, Simmonds & Wong, 2012).

Many species are introduced to new locations deliberately. Introduced species can originate from ornamental stock, as is frequently the case for plants (Hulme et al., 2018), and fish (Chan et al., 2019). Many species are introduced to new areas by unknown individuals who are enthusiasts or released by irresponsible pet owners (Hirsch, N'Guyen & Burkhardt-Holm, 2021). Snakes, and other species with long lifespans like turtles (Dupuis-Desormeaux, Lovich & Whitfield Gibbons, 2022), are more likely to escape or be released when owners no longer want them (Stringham & Lockwood, 2018). Because of their capacity for reproduction in a captive setting, highly fecund species are appealing to people, which also predisposes them to population growth and spread (Street et al., 2023). People may also secondarily spread already introduced species, creating new meta-populations. Red swamp crayfish demonstrate a genetic signature of secondary human introductions, where distance does not explain genetic differentiation in an invasive metapopulation (Yue et al., 2010), and this can only be explained by humans assisting their post-release spread. Animals can also be introduced as part of ranching endeavours, where they are introduced with the intention of harvesting from the population for the pet trade (Episcopio-Sturgeon & Pienaar, 2019). One such example is the case of the Madagascan species, Oustalet's chameleon (*Furcifer oustaleti*), introduced by a reptile importer to an avocado grove behind their property in Miami Dade County, Florida (Gillette et al., 2010). The population has persisted for over a decade despite unusually harsh winters.

Snakes are well adapted to periods of fasting when food is scarce, with some able to dramatically modify their internal anatomy during fasting to reduce energy expenditure (da Mota Araujo et al., 2022). Because they can survive long periods without food and weeks without water (Dezetter et al., 2021), snakes may be introduced to new environments following long journeys as stowaways on ships (Mortensen, Dupont & Olesen, 2008). Some introduced species arrive as part of large populations or are one of many introduction events (Kolbe et al., 2004). This is often the case for marine species transported in the bilge water of boats, where many individuals may be flushed out to form new colonies (Dobrzycka-Krahel & Medina-Villar, 2023). Some vessels make the same trips repeatedly, allowing numerous introduction events. Other animals possess adaptations uniquely suited for spreading to distant environments. Brahminy blind snakes (*Indotyphlops braminus*) are the only known obligately parthenogenetic snake and are found on every continent except Antarctica following introductions through the ornamental plant trade. Every individual of the species is sexually female, and no male has ever been found. The species reproduces asexually. They are likely the result of hybridisation between two other blind snake species. Because they can set up a new population with just one individual, they have been enormously successful, to the extent that their original natural range is uncertain (Khedkar et al., 2022).

There is a substantial body of evidence implicating invasive snake species in the decline of other animals, especially on islands (Piquet & López-Darias, 2021). There were significantly fewer endemic reptiles at sites invaded by Californian king snakes (*Lampropeltis californiae*) on the island of Gran Canaria (Piquet & López-Darias, 2021). On Christmas Island in the Indian Ocean, native reptiles are similarly under pressure from invasive common wolf snakes (*Lycodon capucinus*), and they have been implicated as a major cause of native lizard declines (Mith et al., 2012; Emery et al., 2021). Endemic Ibiza wall lizards (*Podarcis pityusensis*) have been largely extirpated from parts of the island inhabited by Horseshoe snakes (*Hemorrhois hippocrepis*), which arrived through the olive tree industry (Montes et al., 2022). On the island of Guam in the Pacific, brown tree snakes (*Boiga irregularis*) caused serious decline or extirpation of most of the 25 native bird species (Wiles et al., 2003; Rodda & Savidge, 2007). Similarly, the proliferation of Burmese pythons in southern Florida, USA, coincided with the decline of numerous mammal species (Dorcas et al., 2012). Further concerns about their impacts on birds (Dove et al., 2011) have been legitimised by evidence demonstrating significant predation of juvenile wading birds (Orzechowski, Romagosa & Frederick, 2019).

The impacts of Burmese pythons on the ecosystem reach further than the species they directly consume. When the captive animals that formed the introduction escaped captivity, some of them carried a pentastomid parasite (*Raillietiella orientalis*) from their ancestral home in Southeast Asia (Miller et al., 2018). These parasites infect the lungs and have gone on to infect numerous native species (Walden et al., 2020). The infections of native snake species were both more prevalent and higher in intensity than those of Burmese pythons (Miller et al., 2020).

1.2 Control of invasive snakes

Invasive snake species have proven difficult to eradicate. Brown tree snakes on Guam have been the target of eradication attempts for over 20 years, using various methods from hand capture, to searching with dog teams, fumigants, and traps containing dead mice as lures (Clark et al., 2012). The use of dead mice as bait laced with acetaminophen (paracetomol) has also been deemed a humane method of snake control (Clark et al., 2012). Acetaminophen causes lethality by promoting the formation of methaemoglobin, which deprives cells of oxygen and causes unconsciousness and death in a manner analogous to carbon monoxide poisoning (Clark, Clark & Siers, 2017). It has proven more effective than regular minnow-style funnel traps in eradicating snakes (Clark et al., 2012). In Guam where invasive snakes make up a large portion of the biomass and birds have been largely extirpated, air-dropping acetaminophen-laced bait has been used for control (Goetz, Yackel Adams & Siers, 2020). Acetaminophen has also been shown to be effective in causing death to California king snakes, invasive in Gran Canaria (Friebohle, Montgomery & Siers, 2020), but managers stress that eradication is unlikely and note that it was not a panacea for the brown tree snake. Efforts to control the snakes with trapping and manual capture have not stopped the king snake populations from growing (Friebohle, Montgomery & Siers, 2020). They hope that a recently implemented ban on owning snakes from the family Colubridae, such as king snakes, will avoid further introductions to nearby islands. They further recommend enhanced port security, although this has not been implemented (Friebohle, Montgomery & Siers, 2020).

Burmese pythons in Florida have proven similarly difficult to eradicate. Because of the difficulty of attaching traps to the limestone bedrock, trapping has been ineffective (Leatherman, 2022). In addition, the inaccessibility and vastness of the area the pythons inhabit in the Everglades National Park and its vicinity mean that payments for snake bounty hunters turn up relatively few snakes, and each snake takes many hours to find (Leatherman, 2022). Male Burmese pythons are known to follow female scent trails using pheromones (Richard et al., 2019), but experiments with

pheromone attractants which lure male snakes in anticipation of finding a female have so far been unsuccessful in Burmese pythons (Guzy et al., 2023). However, chemically-feminised male snakes have been used to attract males in both red-sided garter snakes (*Thamnophis sirtalis parietalis*) and brown tree snakes (Parker & Mason, 2012; Parker et al., 2018), although they are not as effective as using females. As a result, while using pheromones to attract snakes into traps for removal sounds promising, it is not currently effective. In the meantime, as is the case for many aquatic species in the USA (Green & Grosholz, 2021), focus should now turn to management rather than eradication of Burmese pythons.

1.3 The consequences of genetic isolation

Despite famously successful invasive species, the majority of non-native introductions fail at the first hurdle and never become established (Chapple, Simmonds & Wong, 2012). The genetics of introduced populations are known to be hugely influential in determining success (Lavergne & Molofsky, 2007). Regardless of their mode of transport, most introduced species experience some degree of genetic bottleneck, where diversity is reduced because the founding population represents only part of their original population (Peacock et al., 2009). However, there are cases where introduced species maintain high levels of genetic diversity, sometimes even greater than that of natural populations. In the case of the brown anole (*Anolis sagrei*), multiple introduction events stemming from sources at disparate parts of the range have led to a transformation in their genomic make up, transforming between-population diversity into within-population diversity (Kolbe et al., 2004). Similarly, mixed ancestry from a range of domestic and wild lineages is thought to have contributed to the rapid expansion of feral swine (*Sus scrofa*) in the USA (Smyser et al., 2020). The swine demonstrate how mixed ancestry can benefit introduced species; wild boar are over-represented in the genotypes of feral swine compared to the number of founding animals, which is strongly biased toward domestic pigs. Because of its pervasiveness in the genome, this suggests that wild boar ancestry may contribute strongly to the fitness of the animals in the wild (Smyser et al., 2020).

The lack of domestic pig genomic influence in feral swine is in accordance with captive-adapted genotypes generally performing poorly in the wild (Fraser et al., 2019). Pigs are long-domesticated, but captive breeding can lead to definite reductions in fitness over relatively short timescales. Vancouver island marmots (*Marmota vancouverensis*) lost the ability to distinguish predators within only five generations in captivity (Dixon-MacCallum et al., 2021). Conversely, Mallorcan

midwife toads (*Alytes muletensis*) maintained predatory defence mechanisms following eight generations in captivity (Kraaijeveld-Smit et al., 2006). The extent to which important behaviours and adaptations are lost as a consequence of captivity is likely to be highly variable, and is a key consideration in captive breeding attempts (Kraaijeveld-Smit et al., 2006).

The crucial reason that genetic variability is considered important to introduced species lies in adaptation. Introduced species find themselves in environments with a variety of novel selective pressures where adaptation may prove crucial (Whitney & Gabler, 2008), and this is known to prompt rapid evolutionary change in invading populations (Suarez & Tsutsui, 2008). A lag time following the introduction of invasive species, prior to population expansion, is widely documented (Crooks, 2005), and it has been suggested that this lag time may represent a period of adaptive evolution (Suarez & Tsutsui, 2008). An invasive fly, *Drosophila subobscura*, exhibited local adaptation to temperature after a decade (Huey & Pascual, 2009). Chinook salmon (*Oncorhynchus tshawytscha*) demonstrate that local adaptation following introduction can significantly increase rates of survival and egg production in as little as 26 generations in their introduced range in New Zealand (Kinnison, Unwin & Quinn, 2008). A large-scale translocation project was undertaken for Mojave desert tortoises (*Gopherus agassizii*), where over 9105 tortoises, many former pets, were released into the Large Scale Translocation Site in Nevada, USA (Scott et al., 2020). The survival rates were extremely low, partly due to the project exceeding the carrying capacity of the environment, which was already home to 1450 adult resident tortoises. However, those individuals that survived generally had higher levels of heterozygosity, a measure of genetic variability describing the condition of possessing two different alleles at a genomic marker, than those that died. This demonstrates that tortoises with higher genetically variability were better able to survive after translocation (Scott et al., 2020).

Inbreeding frequently manifests as inbreeding depression. This describes the situation where deleterious alleles, previously relegated to being heterozygotes, emerge as homozygotes following mating with a close relative sharing similar DNA. Inbreeding depression is known to hamper reproductive fitness in some species (Briskie & Mackintosh, 2004). In adders (*Vipera berus*) it has been implicated in deformed and stillborn offspring, and smaller litter sizes in a small, isolated population in Sweden (Madsen, Stille & Shine, 1996). Following the introduction of 20 novel males, there was an increase in the genetic diversity of individuals and an increase in offspring viability (Madsen et al., 2020). Similarly, precipitous decline and resulting lack of genetic variation

was implicated in low reproductive performance for a small population of prairie chickens (*Tympanuchus cupido pinnatus*) in the USA. Reproductive success was improved by translocating 271 chickens from elsewhere (Westemeier et al., 1998). Some species possess life history strategies where the negative consequences of inbreeding depression have been negated, such as frequent sibling mating, where the purging of deleterious alleles has already taken place over generations (Eyer et al., 2018). This may also pre-adapt them for success in biological invasions. But these species are the exception, and small populations are generally associated with the negative consequences of inbreeding.

Despite the effects described above, accurately determining levels of inbreeding depression has been challenging (Kardos et al., 2018), and it is only with the advent of whole-genome sequencing that accurately estimating the heterozygosity of individual animals has become possible. Where before small portions of the genome were examined, modern methods can search for runs of homozygosity across the entire genome (Kardos et al., 2018). This is enormously beneficial for finding runs of homozygosity in animals, a clear signal of inbreeding which can be masked by high genome-wide heterozygosity (Pozzi et al., 2023). This detailed investigation of vertebrate genomes is still in its infancy, and most studies using it involve megafauna (e.g. Bergman et al., 2023). Unfortunately, isolated and small populations are inherently difficult to sample (Jeliazkov et al., 2022), and a lack of data can be a problem when conducting genomic investigations. As a result, gaps remain in our understanding of genetically isolated populations.

1.4 Isolated populations and extinction

Populations with low numbers of individuals are inherently vulnerable to extinction (O'Grady et al., 2004), and even those populations which still survive may be forecast to go extinct based on simulations (Jana et al., 2017). Climate change can be devastating to populations that lack dispersal ability. Tuataras (*Sphenodon punctatus*) have temperature dependent sex determination, with warmer temperatures producing higher numbers of male offspring. An isolated population on North Brother Island, New Zealand, is already showing severe signs of male-bias within the population due to warmer termperatures (Grayson et al., 2014). Because it takes a long time for generations to die-off, populations of long-lived animals like tuataras may appear to be stable for decades despite severe emergent sex biases (Grayson et al., 2014). Unless they can adapt, further climate warming is likely to render that population extinct. Assisted colonisation has been widely used for tuataras,

and future planning should incorporate the consideration that they may become climate migrants (Hunter-Ayad et al., 2021).

Populations of introduced species are fundamentally like naturally isolated populations, and face many of the same challenges. The number of individual animals is crucial to both - as population size best predicts extinction risk in isolated populations, propagule pressure, the number of animals introduced, also predicts the likelihood of establishment success, with larger populations more likely to succeed (Chapple, Simmonds & Wong, 2012). Despite their similarity to assisted migrations, populations which are introduced beyond their natural range limits are generally considered pests to be removed, even if the distance to their natural range is relatively short and could be covered by species with better dispersal capabilities. Warming due to climate change is known to shift animal ranges away from the equator and to higher altitude (Pomara et al., 2014; Lenoir & Svenning, 2015). Introduced species moving northward in Europe could therefore be considered bridgehead populations pre-empting the effects of climate change in anticipation of range shrinkage at the equatorial extreme.

1.5 Plasticity and flexibility

Many reptile species have varied activity windows between populations. Diurnal day geckos (*Phelsuma* spp.) use artificial light to hunt during the night, including in two locations where they are invasive species (Baxter-Gilbert et al., 2021), alleviating them from the confines of their diurnal niche. Behavioural plasticity is therefore known to benefit introduced species but is rarely recorded in introduced populations (Hanshew & Garcia, 2012), and has received less attention than other factors which govern success (Chapple, Simmonds & Wong, 2012).

Behavioural flexibility is one aspect of plasticity. It describes an individual animal's ability to react to alterations in its environment using a variety of behaviours (Wright et al., 2010). Because they have access to a wider suite of possible behaviours when entering a new environment, species which demonstrate behavioural flexibility are more likely to succeed (Wright et al., 2010). Brain size relative to body size has been used as a proxy for behavioural flexibility, and there is a strong link between establishment success of non-native species and brain size for mammals (Sol et al., 2008), and birds (Sol, Timmermans & Lefebvre, 2002). Accordingly, bird species which employ innovative foraging methods are more likely to succeed in novel areas (Sol, Timmermans & Lefebvre, 2002). House sparrows from invasive populations were found to approach and consume

novel foods more willingly than those from a population that was long-established (Martin & Fitzgerald, 2005). Invasive wall lizards were more bold, exploratory, and faster to interact with novel objects than their native congeners (*Podarcis* spp.), reinforcing the link between invasive potential and behaviour (Damas-Moreira et al., 2019). Conversely, an invasive population of skinks demonstrated less exploratory behaviour than their native counterparts (Naimo et al., 2021).

Flexibility may aid species by allowing them to exploit alternative shelters, habitats, or foods. The success of invasive rusty crayfish (*Oronectes rusticus*) is partly attributed to their ability to modify their foraging behaviour in the presence of competitive species, allowing them to access alternative resources (Pintor & Sih, 2009). Crayfish from introduced populations were also significantly more aggressive when competing for food, suggesting selection for competitiveness at the invasion front (Pintor & Sih, 2009). Delicate skinks (*Lampropholis delicata*) are successful invaders while their congener the common garden skink (*Lampropholis guichenoti*) is not, despite similar behaviour and phenotype (Szabo, Damas-Moreira & Whiting, 2020). Delicate skinks tend to be more exploratory in their behaviour, which could account for the difference (Chapple, Simmonds & Wong, 2012). Unfortunately, studies have seldom made comparisons between the behaviour of introduced and native populations of a single species (Szabo, Damas-Moreira & Whiting, 2020). Spatial learning likely also plays a role in introduction success, as invasive green crabs (*Carcinus maenas*) consistently searched areas they had searched least recently, in a process termed "spontaneous alteration". This was not seen from native crabs in a comparative study (Ramey et al., 2009).

The number of behavioural strategies employed within a population may decrease over time as a species adapts locally, but the drivers of this phenomenon are not well understood. Common wall lizards (*Podarcis muralis*) lose antipredator responses after several hundred years isolated from snake predators, likely due to relaxed selection (Durand et al., 2012). Yet after 40 years of isolation from predators, delicate skinks did not exhibit changes in antipredator responses when faced with bird and snake predators (Naimo et al., 2021). Still, the behavioural processes which underpin success in novel environments remain largely unexplored (Damas-Moreira et al., 2019), especially for cryptic species whose behaviour is unknown, such as snakes.

1.6 Thermal tolerance

Surviving extremes of temperature is a useful adaptation for pioneering species in new environments, and tolerance to extremes of climate may evolve during an invasion (Litmer & Murray, 2019). An introduced lizard has been shown to outperform native lizards at cooler temperatures (Caruso, 2021), possibly conveying a competitive advantage. Still, ectothermic animals do not make obvious invaders to temperate climates. While genomic adaptation to survive climatic extremes is well documented, less information exists on the behavioural adaptations that species employ to survive in novel environments. Behavioural thermoregulation, using behaviour such as basking or retreating to cool areas to maintain body temperature (Kearney, Shine & Porter, 2009), can allow animals to counteract suboptimal climates. Invasive flies reduce their active window at lower latitudes to avoid overheating (Huey & Pascual, 2009), remaining hidden during the heat of the day at a time when they would be active in cooler parts of the range. Understanding the thermal tolerance of introduced species can therefore be used to forecast their spread (Christiansen et al., 2015). A suite of traits associated with successful northward dispersal has been recognised in Mediterranean and Ponto-Caspian aquatic invertebrates, and these include an ability to enter diapause or hibernation, and a tolerance of a wide range of temperatures (Dobrzycka-Krahel, Kemp & Fidalgo, 2022). Green crabs show high levels of heat tolerance in their introduced range, with latitude-influenced variation. This greater ability of populations from cool climates to tolerate cold weather is evidence for local adaptive divergence (Tepolt & Somero, 2014).

1.7 Introduced species in urban areas, and commensalism

Urban environments, which cover an increasingly large percentage of the Earth, are frequently the site of animal introductions, owing to their close proximity to people (Borden & Flory, 2021). This also means that species living in urban areas are more likely to be transported to additional novel environments (Chapple, Simmonds & Wong, 2012). Invasive species are often more abundant in urban ecosystems than adjacent natural landscapes (Cadotte et al., 2017), and non-native species richness is predicted to rise in urban areas, exacerbated by the fact that they are hotspots for the transport of people and goods (Tsang, Dyer & Bonebrake, 2019). Accordingly, bird species that frequently live in close association with people are more likely to be successful in new environments (Sol, Timmermans & Lefebvre, 2002). Urban environments are a known driver of rapid evolution in a variety of species (Borden & Flory, 2021), for example Puerto Rican crested

anoles (*Anolis cristellatus*) developed longer hind legs and larger numbers of lamellae (gripping scales of the toes) in response to the need to scale smooth buildings following extensive urbanisation in Puerto Rico (Winchell et al., 2016). These urban-adapted species will likely have an advantage over competitors if they are subsequently transported to novel areas which are similarly urbanised (Borden & Flory, 2021).

1.8 Study species

Aesculapian snakes (*Zamenis longissimus*) are a widespread European species of constricting snake in the family Colubridae, growing to a maximum of two metres long (Edgar & Bird, 2006). They have been introduced to the UK twice in modern times. The first introduction was in Colwyn Bay, North Wales, where breeding snakes were discovered in the 1970s following accidental escape from the Welsh Mountain Zoo (Edgar & Bird, 2006). The second introduction is a population in London on the banks of the Regents Canal, stemming from an introduction event in the 1980s. A third population in Bridgend, South Wales was thought to exist (Clemens & Allain, 2020), but discussion with locals revealed it originated from either a hoax or a miscommunication.

Aesculapian snakes in the UK represent the northernmost extent of the modern range of this species. Because of this, they are an ideal model to study the behaviour of an isolated introduced species. They also exist in two established but non-invasive populations. Because of these multiple populations and an extensive natural range, we can determine the genetic ramifications of early-stage introduction and establishment. Because they have been introduced further north than their natural modern range, they further provide an opportunity to study the behaviour of a predator which must adapt to a cooler environment.

Because Aesculapian snakes have recently arrived in the UK, with two isolated populations, they offer an opportunity to study a nascent introduction. But many snake populations have been naturally isolated for unknown periods, often millions of years. Because of glacial fluctuations during the Pleistocene (2.58 million to 11,700 years ago), some terrestrial populations in Africa became geographically isolated in refugial populations when ice coverage was high (Barlow et al., 2013). For the temperate-adapted and venomous rinkhals (*Hemachatus haemachatus*), it has been suggested that this allowed them to spread northward from southern Africa into Zimbabwe, before subsequently contracting, leaving an isolated population in Nyanga, Zimbabwe. However, the circumstances surrounding the separation of the northern population and populations in the larger

southern range are uncertain, and the extent to which they have diverged is unknown. The rinkhals from Zimbabwe therefore presents an opportunity to determine the biogeographic history and taxonomic status of a long-isolated population of a widespread species.

1.9 Research aims

The aims of this research are as follows:

i) Using radiotelemetry, identify the habitat preferences and behavioural patterns of an introduced snake species living in an isolated population in North Wales.

ii) Using whole-genome sequencing, determine the genetic variation and health of isolated Aesculapian snake populations in the context of multiple natural populations. Additionally, establish the geographic sources of these introductions.

iii) Using the rinkhals from southern Africa as a case study, identify the genetic and morphological effects of isolation within an integrated taxonomic framework.

Chapter 2: A reliance on human habitat features is key to the success of an introduced predatory reptile

Abstract

Understanding the success of animals in novel environments is increasingly important as human-mediated introductions continue to move invasive and non-native species far beyond their natural ranges. Alongside these introductions, inhabited and agricultural areas are spreading, and correspondingly most animal introductions occur in populated areas. Commensal species which can live alongside humans by making use of specific conditions, structures, or prey, have a significant advantage. Introduced mammal species often use anthropogenic features in their environment and demonstrate a higher tolerance of human disturbance, but the importance of human features of the landscape remains understudied in ectotherms. The Aesculapian snake (*Zamenis longissimus*) is an ectotherm which has been introduced beyond the northern extremities of its natural range. To understand the persistence of this species, we radio-tracked snakes daily over two active seasons and investigated their home range size using Autocorrelated Kernel Density Estimators (AKDE). Using AKDE-weighted Habitat Selection Functions we identified preferences for habitat features in a mosaic of habitats, and we used Integrated Step Selection Functions to further explore how these features influence movement. We revealed a particular preference for buildings in male snakes, while females preferred woodland. We demonstrate that the success of an ectothermic predator is likely tied to a willingness to use human features of the landscape.

2.1 Introduction

Through time, the areas inhabited by species are pushed and pulled in many directions by climate, habitat changes, interactions with other species, and human transport. The latter has become a particularly powerful force, introducing species to distant, novel habitats where they are exposed to a range of entirely different physical and climatic processes as well as different biotic interactions to those in their native ranges. Not all species are equally successful when faced with such pressures. Identifying the key characteristics underpinning the success or failure of species in novel environments can help predict winners and losers in a future where all animals are facing increased challenges (Moyle & Marchetti, 2006). Through the tracking of animal movements, we can

understand traits which are key to success in unfamiliar locations, such as colonisation and dispersal abilities, and habitat requirements (Bubb, Lucas & Thom, 2002; Bartoszek et al., 2021; Kays et al., 2022). Using these insights, we can begin to explore how introduced species both adapt to and impact their new habitats.

Many introduced species demonstrate an ability to utilise human features, including fragmented landscapes and anthropogenic structures, to travel and hunt (Andersen et al., 2017), and introduced animals have generally demonstrated greater tolerance of human disturbance than their native counterparts (Bielen et al., 2016). Being able to use these anthropogenic features is advantageous because non-native species are most likely to be introduced in or near human-impacted habitats, and it is frequently generalist species that capitalise on these disturbed habitats and make good invaders (Marvier, Kareiva & Neubert, 2004). While much of the available literature focuses on terrestrial endotherms and their adaptability in the face of human dominated landscapes, comparatively little focuses on ectotherms – particularly snakes (Liu et al., 2020). This is despite several prominent examples of introduced snakes (albeit in more natural landscapes) having considerable impacts on the native fauna (Rodda & Savidge, 2007; Dorcas et al., 2012).

Snake introductions are not limited to warm climates. Aesculapian snakes *Zamenis longissimus* (LAURENTI, 1768) are a constricting species in the family Colubridae. Native to mainland Europe, they range from France in the West to Iran in the East (Musilová, Zavadil & Kotlík, 2007). The species previously occupied a larger part of Northern Europe, only recently going extinct in Denmark during the early 1900s (Allentoft, Rasmussen & Kristensen, 2018), with remaining relict populations in Germany, Switzerland, the Czech Republic, and Poland. Aesculapian snakes have been introduced twice to the UK in modern times (Allentoft, Rasmussen & Kristensen, 2018). Snakes were introduced to Colwyn Bay, North Wales following an escape from the Welsh Mountain Zoo in the 1970s. This population represents the northernmost modern population of Aesculapian snakes, and its persistence here raises the question of how a species can expand northward, despite the constraints of ectothermy.

The overall aim of this study was to investigate the spatial ecology of an introduced predator, adapted to a warmer climate but existing in cool North Wales. We used radiotelemetry to determine how these animals use their new range, and we set out to discover the home range and space use requirements of both male and female Aesculapian snakes. Our second goal was to discover the habitat preferences of this species, which is crucial to understanding its survival. As we had

experienced snakes entering buildings and vegetation piles, we hypothesised that snakes may be reliant on human features of the landscape. Finally, we were keen to know the dispersal capabilities of this species. We wanted to learn which habitats represent pathways to mobile snakes, allowing us to infer their likely routes should this population spread into the surrounding area. We hypothesised that hedgerows, as linear features in the habitat, would represent pathways for snakes travelling longer distances.

2.2 Methods

2.2.1 Study area and animals

The study site (approximately 1.72 km², Figure 2.1) is located between the town of Colwyn Bay and the village of Mochdre, North Wales, UK (53.28–53.29°N, -3.74–3.76°W). The area consists of a mosaic of habitats, including housing, with meadows and pastures separated by hedgerows. Most pastures are grazed periodically by sheep and cattle. The site also includes the Welsh Mountain Zoo covering an area of 0.15 km^2. The Zoo grounds are a mixture of enclosures, footpaths, and landscaped areas, representing highly disturbed habitats, as well as a patch of woodland in the north corner. Most of the animal faecal matter and vegetation waste is transported to a large dung heap in the southeastern corner of the zoo. Patches of deciduous forest and small patches of gorse scrub are scattered over the entire study site, and a small patch of woodland to the east of the Zoo was in the process of being removed for a new development in 2022. Roads are found throughout, with busy roads surrounding the zoo and the connecting meadows and pastures, with a dual carriageway (A55) at the site's northernmost extremity.

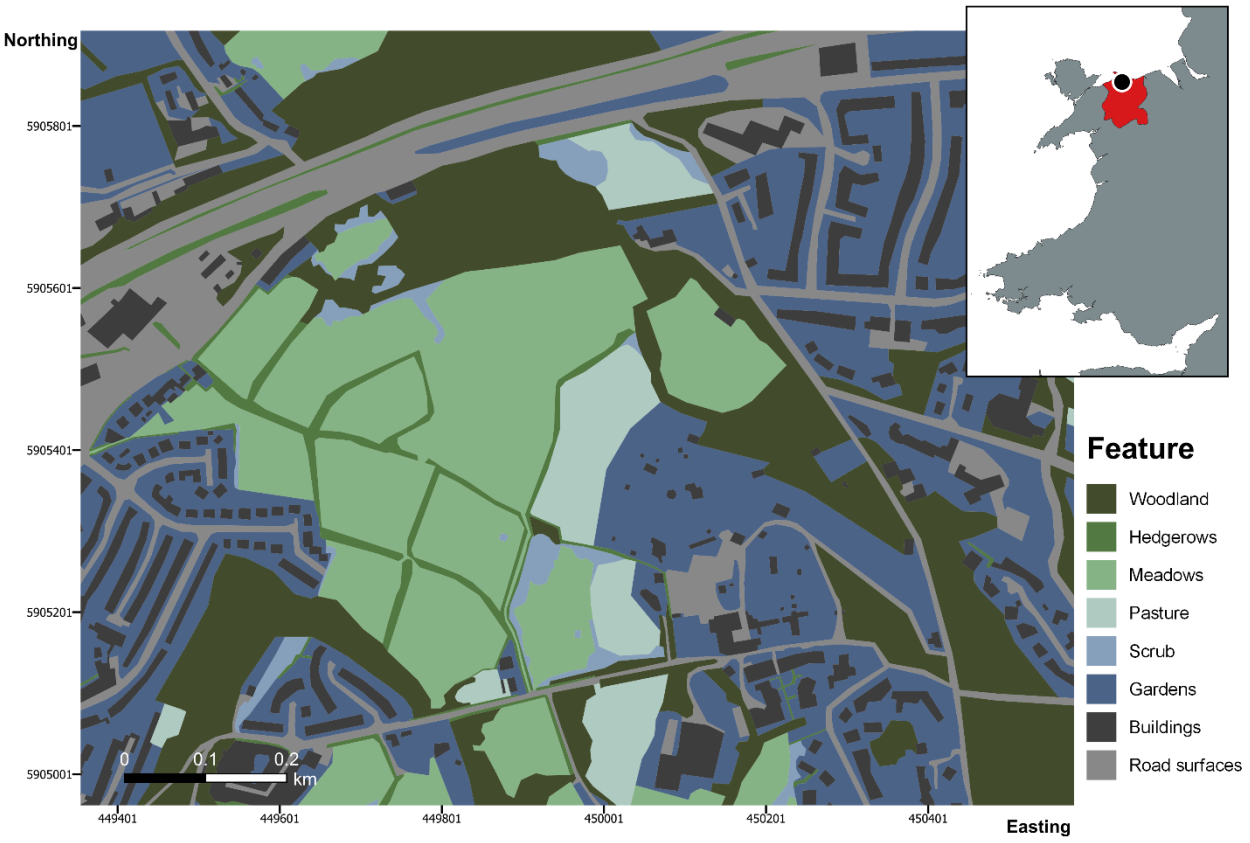

Figure 2.1 Map of the study area in Colwyn Bay showing the habitat classifications used in the habitat selection analyses. Inset map shows location of the study site in Wales, UK, with Conwy County highlighted red. Map created using QGIS (QGIS Development Team, 2023).

We implanted 21 adult Aesculapian snakes with radio transmitters during June – October 2021 and May – September 2022 (see Table 2.1 for tracking and capture summary). Our sample consisted of 13 males and eight females. Snakes were caught by hand, either during dedicated surveys, opportunistically, during radio tracking activities, or following notification by members of the public. Two tracked individuals were caught by keepers at the Welsh Mountain Zoo during their daily activities. Because of the difficulty of finding Aesculapian snakes, which in this population takes approximately eight hours of searching per adult snake found, we radio-tracked any available adult snakes with sufficient body diameter to carry a transmitter. Snakes were transported to Bangor University for transmitter application, and we collected morphometric data including snout-vent length (SVL), tail length (TL) and mass (Table 2.1 and Table S2.1). We attempted to minimise the

time snakes were kept in captivity for implantation procedures (n = 21 implantations, mean = 7.34 ± 6.7 days held, range = 1 – 23 days). Snakes were held in 70L plastic boxes (710 x 545 x 190 mm) in a temperature-controlled room at 21°C with suitable refuge and water provided *ad libitum*.

Table 2.1: Tracking and capture summary for all 21 tracked snakes. Abbreviated capture types are mating with a tracked snake (mating), notification from the public (notification), and dedicated survey (survey). We did not record snout-vent length (SVL) for F203 in error.

ID	Mass (g)	SVL (mm)	Capture type	Start	End	Days	Fixes	Fate
F050	208	766	Opportunistic	18/06/2022	16/07/2022	28	57	Transmitter out of battery
F142	233	668	Notification	04/06/2021	07/07/2021	33	56	Dead - car strike
F158	207	692	Opportunistic	25/06/2022	14/08/2022	50	101	Transmitter out of battery
F159	266	845	Opportunistic	19/06/2021	19/08/2021	61	101	Dead - eaten by M137
F177	237	818	Opportunistic	22/07/2021	20/10/2021	90	128	Transmitter out of battery in 2021. Dead - car strike in 2022
F203	211	-	Zookeeper	14/05/2022	18/07/2022	65	216	Transmitter out of battery
F212	343	785	Mating	31/05/2022	04/06/2022	4	23	Transmitter malfunction
F219	322	872	Opportunistic	30/06/2022	12/07/2022	12	24	Transmitter malfunction
M031	383	960	Opportunistic	08/06/2022	11/07/2022	33	164	Dead - car strike
M073	395	1069	Survey	05/06/2021	14/06/2021	9	17	Dead - buzzard predation
M074	399	1089	Opportunistic	16/07/2022	15/08/2022	30	144	Transmitter out of battery
M137	512	1178	Survey	04/06/2021	08/10/2021	126	210	Transmitter out of battery
M139	295	956	Survey	04/06/2021	07/09/2021	95	166	Transmitter out of battery
M149	474	941	Opportunistic	05/06/2021	12/07/2021	37	62	Dead - mammal predation
M154	398	971	Opportunistic	18/06/2022	31/08/2022	74	316	Study period ended

M178	440	1052	Opportunistic	29/07/2021	20/10/2021	83	111	Study period ended
M180	463	1011	Notification	06/08/2021	02/10/2021	57	95	Study period ended
M202	498	1200	Survey	12/05/2022	31/08/2022	112	476	Study period ended
M209	475	982	Zookeeper	16/05/2022	31/08/2022	107	438	Study period ended
M217	235	801	Opportunistic	15/06/2022	01/07/2022	17	82	Transmitter out of battery
M218	522	1115	Opportunistic	25/06/2022	31/08/2022	67	242	Study period ended

Depending on their size, snakes were implanted with 1.2, 1.4 or 1.6 g Holohil BD-2T radio transmitters (Holohil Inc, Canada) following Reinert & Cundall (1982), using isoflurane anaesthetic and butorphanol analgesia, with an internal securing stitch (Alworth, Hernandez & Divers, 2011). Post-implantation, snakes were kept overnight at a constant 21°C and released the following day. One snake (F159) was held for an additional day post-surgery to ensure wound closure. Snakes were released at their exact point of capture in dry conditions warmer than 14°C. The only exception was M180 who was caught basking on top of a hedge in a residential garden. The homeowner requested we release the snake a short distance from their garden, and we released him approximately 30 m away in a hedgerow. As snakes appeared to be behaving normally immediately after release, we began tracking the following day and did not discard any data.

2.2.2 Radio tracking procedures

Snakes were manually located twice daily as part of two daily tracking rounds, the first beginning at 10:00 and a second at 14:00. As we were a small team tracking many snakes, the timings of the tracks were not precise, but animals were usually tracked once in the morning and once in the afternoon, and almost always twice daily. See Figures S2.1 and S2.2 for tracking periods and time lags. In 2022 a subset of seven male snakes were located five times daily. These snakes were tracked in sessions beginning at 0900, 1100, 1300, 1500 and 1700. We wanted to see what type of habitat the snakes use when they are moving, and we intended the greater tracking frequency to capture more points in the movement pathway. One female (F203) was tracked five times daily between 14/05/2022 and 14/06/2022, before switching to twice daily. After 20/08/2022, we began tracking the four male snakes with remaining transmitter battery twice daily (Figure S2.1). When we located a snake, we recorded the temperature, humidity, and location, and noted any behaviour.

We used a Garmin GPSMAP 64S to collect GPS locations. To avoid disturbing the snake and thus influencing their behaviour, we attempted to keep 10 metres between observers and tracked snakes, but within the confines of narrow gardens this was not always possible.

2.2.3 Snake home range estimation

Since the inception of radio telemetry using very high frequency (VHF) transmitters and receivers, home range estimation measures such as minimum convex polygons and kernel density estimators have been used in determining the home range of animals (Silva et al., 2020). These methods assume independent and identically distributed data, and do not account for autocorrelation, where data points close in time are also close in space (de Solla, Bonduriansky & Brooks, 1999). This was generally acceptable for studies of animals which exhibit frequent movement, especially because of the large time lag between points that result from manually relocating the animal using VHF telemetry. However, for studies of animals which spend long periods inactive, such as snakes, autocorrelation presents a major concern and renders traditional home range estimators unreliable (Silva et al., 2020). The novel approach of Autocorrelated Kernel Density Estimation (AKDE) overcomes the limitations of traditional estimation techniques in that it accounts for autocorrelation, and these models present the additional benefit of providing confidence intervals for the estimates of home range size provided (Silva et al., 2022).

To implement this approach, we used the *ctmm* package (Calabrese, Fleming & Gurarie, 2016) and R v4.2.1 (R Core Team, 2022, p. 4) to fit continuous-time stochastic process movement models to our snake movement data. We first checked individuals for range residency by calculating the semi-variance function and visualising it using variogram analysis (Fleming & Calabrese, 2021). We removed individuals that did not demonstrate range-residency from the home range analysis. We fitted multiple movement models and used AICc to identify the model best fitting the autocorrelation structure for each snake. These were either the Ornstein-Uhlenbeck (OU) model where the animal exhibits Brownian motion restricted to a finite home range, or the OUF model with continuous-velocity motion restricted to a finite home range, or Independent Identically Distributed (IID). These prototype models are either isotropic, where there is equal diffusion in every direction, or anisotropic, where diffusion varies depending on direction (Calabrese, Fleming & Gurarie, 2016). For optimal performance we estimated autocorrelation and covariance bias using pertubative hybrid residual maximum likelihood (pHREML), accounting for both small absolute

sample size and small effective sample size (Fleming et al., 2019). Absolute sample size refers to the total number of times the animal was located during the tracking period, while effective sample size is the entire tracking duration divided by how long it takes, on average, for the animal to cross its linear home range. We then fit AKDEs using the guidance provided by (Silva et al., 2022). We used weighted AKDEc and pHREML to estimate home range size, which reduces oversmoothing of range limits, particularly in cases with small effective sample sizes (Silva et al., 2022). It also helps to address irregular sampling. Silva et al. (2022) recommend pHREML and AKDEc for effective sample sizes (range crossings) below 20, which applied to most individuals. Parametric bootstrapping can also be used to reduce estimation error, and we used ctmm.boot in the *ctmm* package to apply parametric bootstrapping to individuals with low effective sample sizes (Silva et al., 2018; Thompson et al., 2021).

2.2.4 Space use

In recent years there has been a rise in the use of dynamic Brownian Bridge Movement Models (dBBMMs) to estimate the home ranges of animals, but these estimators are not suitable for this purpose (Alston et al., 2022b). As these models are occurrence distribution estimators and not range distribution estimators, these models use animal locations over time to interpolate where animals might have been during a tracking period, rather than extrapolating to their entire range (Alston et al., 2022b). The use of dBBMMs to estimate home range size therefore results in dramatic underestimates, especially for short tracking durations; however, it does allow for comparison with other studies and allows an impression of snake spatial ecology during the study period.

As many snakes were unlikely to have sufficient tracking durations to facilitate home range estimation, we use estimates of space use derived from dBBMMs. We estimated snake space use using the R package *move* v4.1.10 (Kranstaber, Smolla & Scharf, 2022). As snakes were generally active for a few days before spending between a few days and a week inactive, we specified a moving window of 11. With our twice daily tracking regime, this allowed us to detect variations in the behavioural state of snakes over a six day period (D'souza et al., 2021). We chose a margin size of three to allow detection of active vs inactive states (D'souza et al., 2021), and used the mean GPS error of our snake locations as the telemetry error. We used two daily tracks to model space use across all individuals.

2.2.5 Habitat selection

All analyses were conducted using R v4.2.1 (R Core Team, 2022). For all habitat use analyses, we used custom shape files of habitat types (Figure 2.1), created in QGIS (QGIS Development Team, 2023). This map initially contained nine habitat types: meadow, pasture, hedgerow, woodland, scrub, gardens, buildings, bare ground, and roads. We combined roads with small areas of bare asphalt (bare ground) in the surrounding area to create 'road surfaces'. Meadow was defined as ungrazed areas with a sward height > 20 cm, although some meadows were grazed by cattle in late Summer 2022. Pasture was defined as fields with grass which was kept short either by livestock or frequent mowing. Using modified code from Smith et al (Smith et al., 2021) and Hodges et al (Hodges et al., 2022), we converted our raster layers into layers with continuous gradients denoting the Euclidean distance to habitat features. These layers were then inverted to avoid zero-inflation. By inverting the layers, previously near-zero values become higher values, mitigating potential model bias associated with high numbers of zeros. It also ensured that the resulting outputs were easy to interpret, as positive values indicate selection.

2.2.6 Integrated Resource (Habitat) Selection Functions

Traditional resource selection function analyses, now termed habitat selection functions (Fieberg et al., 2021), do not allow for autocorrelated data and assume independence between each point at which an animal is located. However, for high-resolution GPS location frequencies, or for animals which move infrequently, points are not independent from each other. To avoid the need to thin data to ensure independence of points, we utilised habitat selection functions informed by our AKDEs, which down-weight autocorrelated points rather than discarding them (Alston et al., 2022a). We used rsf.fit within the *ctmm* package (Fleming & Calabrese, 2021) to fit integrated resource selection functions to our snake tracking data, with simultaneously estimated spatial constraints. We used the Monte Carlo numerical integrator for likelihood evaluation, with a numerical error threshold of 0.05 for the parameter estimates and log-likelihood.

2.2.7 Integrated Step Selection Functions

We used Integrated Step Selection Functions (ISSF) to analyse how habitat types influenced the movements of Aesculapian snakes at the population scale. This allowed us to incorporate the movements of all tracked individuals, even those with short tracking durations that did not

demonstrate range-residency. We split the data into male and female snakes and adapted code from Smith et al (2021) and Muff et al. (2019) to run mixed conditional Poisson regression models essentially operating as "population-level ISSFs" on our twice-daily tracking data. The first and third daily tracks of snakes which had been tracked five times daily were used to ensure comparability with our twice daily data. Using the *INLA* package v22.05.07 (Rue, Martino & Chopin, 2009) we ran a population-level ISSF to ascertain either association or avoidance of our eight habitat classifications. We removed zero distance steps (non-moves) from this analysis. We generated 200 random points per move, for step length we used a Gamma distribution and for turn angle Von Mises (Smith et al., 2021; Hodges et al., 2022), to facilitate comparison between locations the snakes selected and locations they did not. We created eight single-factor models, one for each habitat type, and all models included the interaction of turn angle and step length. As per Muff et al. (2019) the stratum-specific random effect of step was set to 0.0001. In keeping with Smith et al. (2021), we utilised a Penalised Complexity prior, PC (1, 0.05) for the random slope, which was individual, and uninformative normal priors were used for the fixed effects. Using the *INLA* package v22.05.07 (Rue, Martino & Chopin, 2009), we used nested Laplace approximations in fitting these models.

2.2.8 Five daily tracks

For the male snakes tracked five times daily, we ran both individual ISSFs as well as the "population-level ISSFs" described above to investigate whether Aesculapian snakes demonstrated attraction to or avoidance of habitat types at the scale of the entire population. For individual selection, we created ISSFs using *INLA* package v22.05.07 (Rue, Martino & Chopin, 2009), filtering steps using a resample rate of two hours with a tolerance of four hours to avoid bias from unexpected overnight movements. We created nine single-factor models, one for each habitat type, and one representing the null model. The individual habitat models also included the interaction of turn angle and step length, while the null model only included the interaction of step length and turn angle. Otherwise, we used the same settings as described above. We used AIC scores to determine which features most strongly influenced the habitat selection of Aesculapian snake individual movements from our ISSF. Models with the lowest AIC score or scores < 2 greater than the lowest were considered to have the largest influence.

2.2.9 Seasonality and shelter locations

We used two measures to determine seasonal behaviour changes in Aesculapian snakes: mean daily displacement and dBBMM-derived motion variance (Hodges et al., 2022). We use dBBMMs to visualise the space use of tracked snakes over the tracking period and ascertain areas of high use and re-use that are of particular significance to Aesculapian snakes. We also use the estimates of motion variance they provide to determine periods of heightened activity during the tracking period (Hodges et al., 2022).

Although mean daily displacement (MDD) has limitations, we were unable to estimate speed or distance travelled using continuous-time movement models [10], likely due to the relative infrequency of our data points compared to high-resolution GPS data. Despite the shortcomings of MDD (Rowcliffe et al., 2012), we kept to a strict tracking schedule of at least two tracks per day for all individuals, allowing increased confidence in the MDD estimates (Figure S2.2). To investigate seasonality, we filtered the first and third daily tracks of snakes which received five tracks per day to allow comparison between all individuals. We summed the movement distance across these two daily tracks to create our values for daily displacement. We opportunistically collected observational data on the breeding behaviour of this population during tracking activities, which allowed us to better inform the dates of the mating and egg-laying seasons.

To understand areas snakes used for long periods, we first created move objects in R using twice daily tracking data for all 21 individuals with the package *move* v4.1.10 (Kranstaber, Smolla & Scharf, 2022). Using the *recurse* package v1.1.2 (Bracis, Bildstein & Mueller, 2018), we set a radius of five meters around each location a snake visited. We recorded the amount of time each snake spent in the same location for multiple fixes as time spent stationary. Finally, we used the recurse package to visualise places the snakes had spent long periods, and recorded what these locations were by overlaying the GPS locations on a map. We verified the locations using our behavioural notes.

In line with the STRANGE framework (Webster & Rutz, 2020), we recognise that there may be bias in the trappability and self-selection of our sample of snakes. Snakes were frequently near people or their dwellings when captured. Seven of the 13 male snakes were captured at different times under one tarpaulin covering a wood pile in the garden of a residential home, while three of

eight females came from one garden containing multiple mature compost heaps. As many snakes were found near each other, there is also a higher likelihood they are related.

All figures were created using R v4.2.1 (https://r-project.org/). For data manipulation we used the *amt* package v0.1.7 (Signer, Fieberg & Avgar, 2019) and the *dplyr* package v1.0.10 (Wickham et al., 2022). For data visualisation we used *ggplot2* v3.3.6 (Wickham, 2016). For visualising movement data and creating tracks we used *move* v4.1.10 (Kranstaber, Smolla & Scharf, 2022).

2.3 Results

2.3.1 Movements

We tracked 13 adult male and eight adult female Aesculapian snakes between June and October 2021 and May and September 2022. The average tracking duration was 56.67 ± SE 7.85 days (range 4 – 126 days). We collected 3232 total snake locations, including 947 total relocations. The mean daily displacement (MDD) for six male snakes on the twice daily tracking regime was 38.45 ± 21.33 m, and for the seven males on the five times daily regime it was 52.34 ± 26.43 m. The MDD of eight females tracked twice daily was 26.14 ± 18.55 m.

2.3.2 Snake home ranges

Eight males and three females were found to have stable home ranges using variogram analysis (Figure S2.3 and S2.4). The remaining ten individuals did not reach an aysmptote (Fleming et al., 2014). For these individuals, the short tracking durations meant that there was insufficient information to inform our home range estimation model, and they were excluded from Autocorrelated Kernel Density Estimation (AKDE). Full model results for all individuals can be found in Table 2.2. The mean effective sample size for snakes included in the AKDE analysis was 12.92 ± 8.57 (range 4.29 – 35.57). These figures are relatively low and demonstrate that using the pHREML method and weighting the AKDEs was justified (Silva et al., 2022). The mean 95% AKDE estimate for home range size for the three range resident females was 23.32 ± 29.74 ha (range 0.28 – 65.31 ha). For eight range resident males it was 28.86 ± 28.42 ha (range 2.05 – 92.16 ha). For range resident snakes (Figure 2.2 and 2.3), the top models were Ornstein-Uhlenbeck (OU) or Ornstein-Uhlenbeck foraging (OUF). Two individuals, F159 and M218, had low effective sample sizes of six and 4.3 respectively, with pHREML bias in the order of 3% or higher (Table

2.2), and we applied parametric bootstrapping to these individuals. All models reflected elliptical home ranges (anisotropic) except for F158 which was more circular (isotropic). The traditional KDE approach of using Independent Identically Distributed (IID) points proved ineffective and the IID models had high dAICc values (Table S2.2).

Table 2.2: Results of the snake AKDE home range analysis. Effective sample size refers to the number of times the animal crossed its home range during the tracking period, while absolute sample size is the total number of observations (fixes). Contour area estimates of home range for individuals presented in hectares. F159 and M218 had parametric bootstrapping applied to reduce error on their home-range estimates and the estimates displayed are post-bootstrapping.

ID	AICc top model	Effective sample size	Absolute sample size	95% AKDE lower CI (ha)	95% AKDE estimate (ha)	95% AKDE upper CI (ha)	pHREML bias	Parametric bootstrap bias
F158	OU	8.72	102	1.97	4.37	7.72	0.013	-
F159	OUF anisotropic	4.71	102	20.10	64.75	135.03	0.028	0.0096
F177	OU anisotropic	35.57	126	0.19	0.28	0.38	0.001	-
M031	OU anisotropic	19.44	165	1.24	2.05	3.05	0.003	-
M137	OU anisotropic	9.36	209	9.98	21.44	37.20	0.011	-
M139	OU anisotropic	8.69	167	7.83	17.41	30.75	0.013	-
M154	OU anisotropic	7.66	302	4.54	10.75	19.59	0.017	-
M180	OU anisotropic	14.89	96	1.46	2.61	4.10	0.005	-
M202	OU anisotropic	8.83	457	23.37	51.57	90.74	0.013	-
M209	OU anisotropic	18.74	423	19.72	32.89	49.37	0.003	-
M218	OU anisotropic	4.08	192	100	120.04	261.50	0.054	0.0147

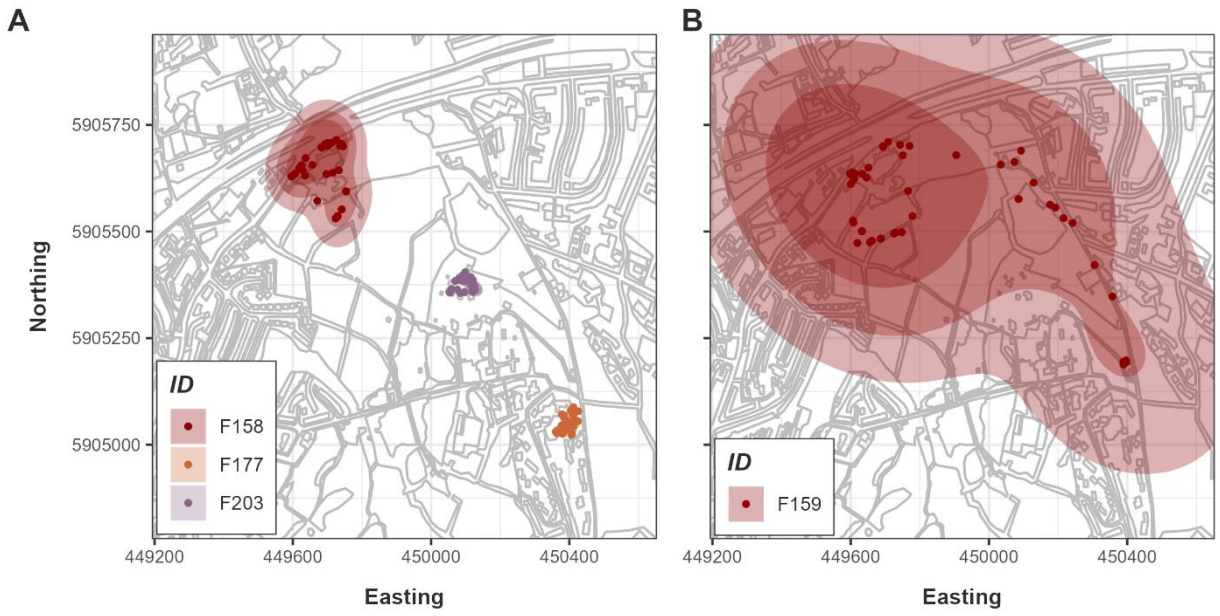

Figure 2.2: AKDE home ranges for tracked female snakes that demonstrated range-residency. A) F158, F177, and F203. B) F159. Darkest shading in the centre represents the lower confidence interval for the 95% home range contour, medium shading is the 95% contour, and the lightest shading is the upper confidence interval. Points represent location data of each animal. While F203 did not demonstrate range residency, we plot the data here for illustration purposes as she was tracked for a relatively long period of 65 days.

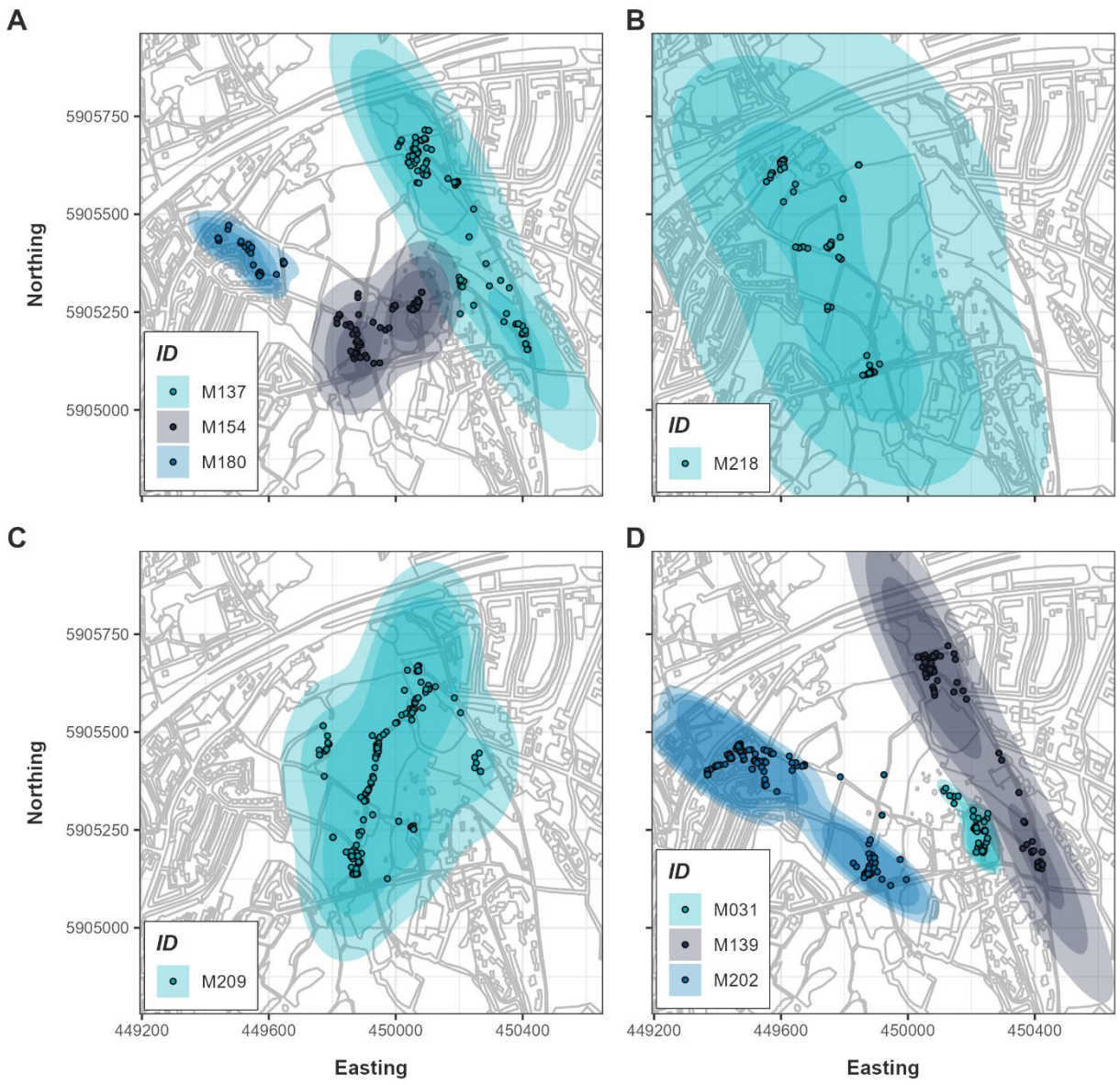

Figure 2.3: AKDE home ranges for tracked male snakes which were range resident. A) M137, M154, and M180. B) M218. C) M209, and D) M031, M139, and M202. Darkest shading in the centre represents the lower confidence interval for the 95% home range contour, medium shading is the 95% contour, and the lightest shading is the upper confidence interval. Points represent location data of each animal.

2.3.3 Snake space use

We estimated space use using dBBMMs (Table 2.3). We excluded three snakes with tracking durations less than 14 days (F212, F219, and M073) from the following means as their space use estimates would likely skew the results. For females, the mean area of the 95% confidence occurrence distributions was 2.09 ± 3.79 ha (range = 0.02 – 10.51 ha). The mean area of the 95% confidence occurrence distributions for males was 6.34 ± 7.10 ha (range 0.46 – 20.72 ha).

Table 2.3: dBBMM occurrence distributions and mean daily displacement (MDD) for the Aesculapian snakes tracked in this study.

ID	90% (ha)	95% (ha)	99% (ha)	Tracking duration (days)	Total distance moved (m)	MDD (m)	Maximum daily distance (m)
F050	0.02	0.05	0.16	28	216.20	7.74	56.78
F142	0.02	0.02	0.04	33	264.26	8.05	58.09
F158	0.55	1.30	3.23	50	1028.29	20.57	92.10
F159	5.91	10.51	18.42	61	3673.04	60.38	364.43
F177	0.21	0.32	0.59	90	782.51	8.68	44.67
F203	0.19	0.32	0.92	65	1282.96	19.70	72.06
F212	0.07	0.09	0.13	4	157.93	38.04	64.73
F219	0.01	0.01	0.02	12	555.29	46.00	244.47
M031	0.63	1.03	1.97	33	1135.56	34.20	129.11
M073	9.03	12.48	20.82	9	698.28	78.71	206.39
M074	0.01	0.01	0.02	30	179.99	5.97	64.96
M137	1.82	4.51	14.49	126	3894.85	30.95	382.88

M139	2.27	7.21	22.72	95	3903.90	41.10	559.30
M149	1.56	2.76	6.41	37	1706.27	46.37	336.90
M154	1.79	3.67	7.57	74	2427.55	32.70	107.16
M178	0.29	0.46	0.87	83	999.82	12.04	255.31
M180	1.31	2.13	4.48	57	1225.27	21.54	125.12
M202	5.55	10.75	25.43	112	7935.06	70.85	566.25
M209	14.10	21.00	35.59	107	7387.04	69.03	365.16
M217	0.35	1.85	5.27	17	1477.92	86.94	383.95
M218	11.94	20.72	43.71	67	4469.91	66.73	586.54

2.3.4 Individual habitat selection via AKDE-weighted RSF

We had sufficient data to inform range-residency and perform the habitat selection analysis on three female and eight male Aesculapian snakes. Parameter estimates for resource selection are visualised in Figure 2.4. The results for the remaining snakes are in Figure S2.5, and all results presented in Table S2.3. In male snakes, buildings were the most commonly selected-for habitat type with five of eight individuals demonstrating positive selection for buildings and two more strongly suggesting it without definitive evidence (Figure 2.4). M137 and M139 showed a preference for woodland, while M202 and M209 were associated with pasture. One chose gardens (M180), one selected meadows (M202), and similarly only (M154) showed a preference for hedgerows. For females, F159 showed avoidance of pasture. We were unable to determine individual preference or for any habitat type for females in this analysis. With regards to males avoiding habitat types, three individuals (M031, M180 and M202) avoided roads, while M202 avoided woodland.

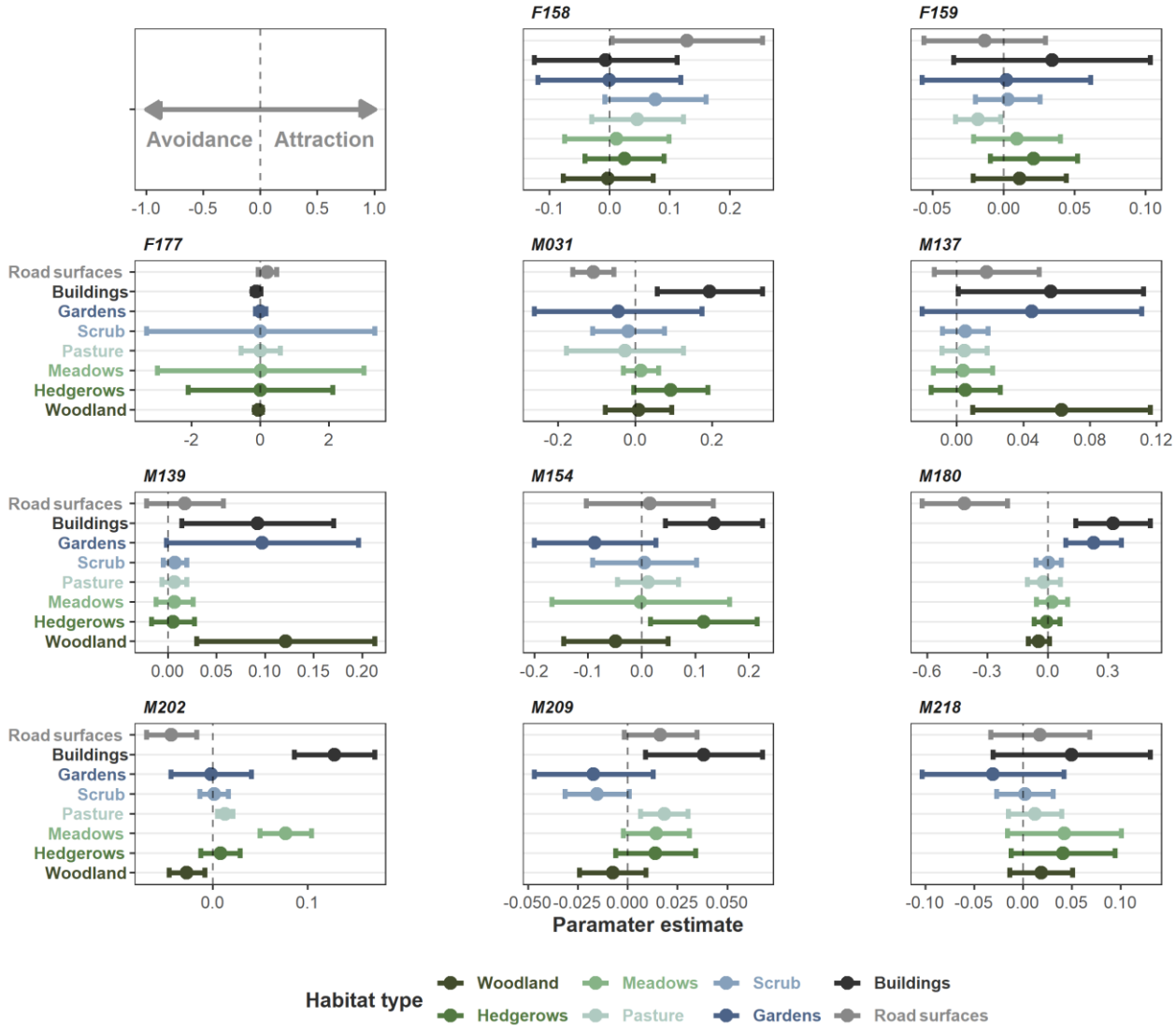

Figure 2.4: Coefficients from the weighted AKDE habitat selection functions for range resident snakes. Each plot displays the habitat selection of one individual snake. Positive values indicate selection for a habitat type, while negative values indicate avoidance. Error bars represent 95% confidence intervals.

2.3.5 ISSF at the population scale

The ISSF analysis suggests woodland is of importance for females at the population-level (Figure 2.5). Males demonstrated habitat generalism, showing selection for meadows, scrub, road surfaces, and possibly hedgerows, but appearing to select areas nearer to buildings and gardens most strongly

(Figure 2.6). We were unable to detect differences in step length associated with different habitat types for either females or males at the population scale (Figure S2.6 and S2.7).

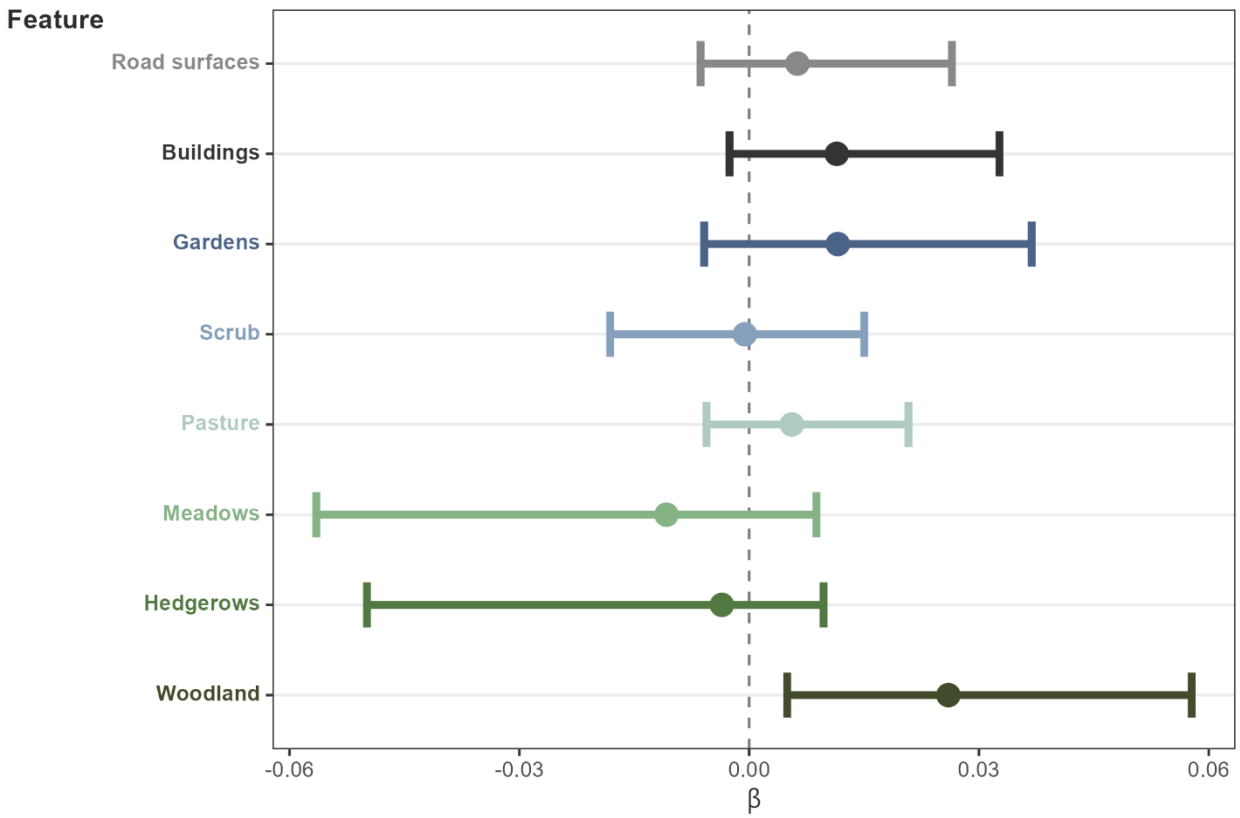

Figure 2.5: Results of the ISSF analysis at the population-level for female snakes (n = 8). Bars represent 95% confidence intervals. Positive values indicate selection toward a particular habitat type, while negative values indicate avoidance.

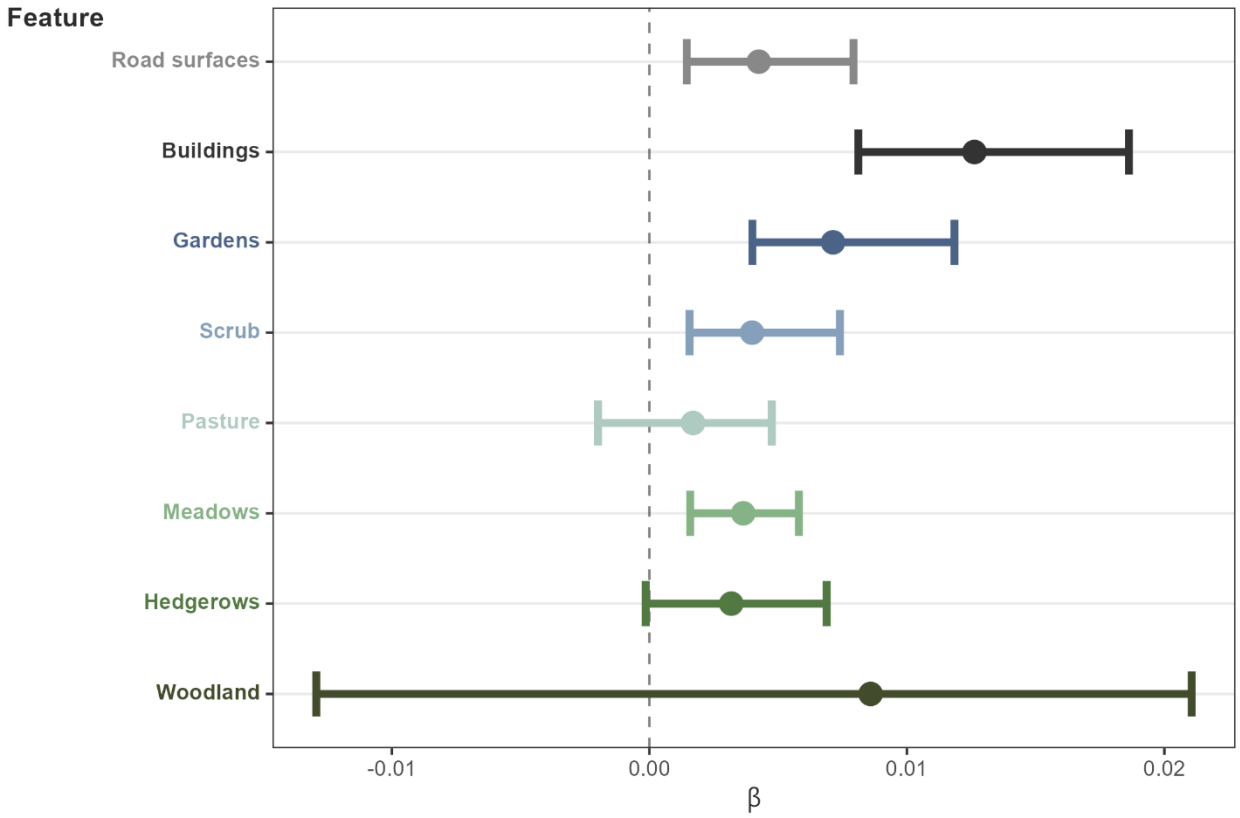

Figure 2.6: Results of the ISSF analysis at the population-level for male snakes (n = 13). Bars represent 95% confidence intervals. Positive values indicate selection toward a particular habitat type and negative values indicate avoidance.

2.3.6 Step length from five times daily tracking

We included the data from six of the seven males that were tracked five times daily, excluding M074 who spent 29 of 30 tracking days stationary. We investigated the influence of habitat type on the step lengths of individual snakes (Figure 2.7). For M202, shorter steps were associated with buildings and roads. For M218, both proximity to pasture and hedgerows were associated with shorter steps.

We also determined which features influenced the habitat selection of snakes. The top model was pasture for M218. Gardens were top or second for M154 and M217. Hedgerows were top or second for M154 and M209, while M207. M031, M154, and M202 were influenced by buildings (Table S2.4 and Figure S2.8). Model selection results do not necessarily equate to definite selection or avoidance, however. At the individual level, two snakes, M154 and M202, preferred to be closer to

meadows and pasture. M218 showed a positive association with hedgerows and scrub. None of the remaining males tracked five times daily showed definitive habitat association in this analysis. We also ran the population-level analysis for this group, who demonstrated a preference for hedgerows, buildings, and scrub (Figure S2.9). There was no significant influence of habitat type on step length using the population-level analysis with this group (Figure S2.10).

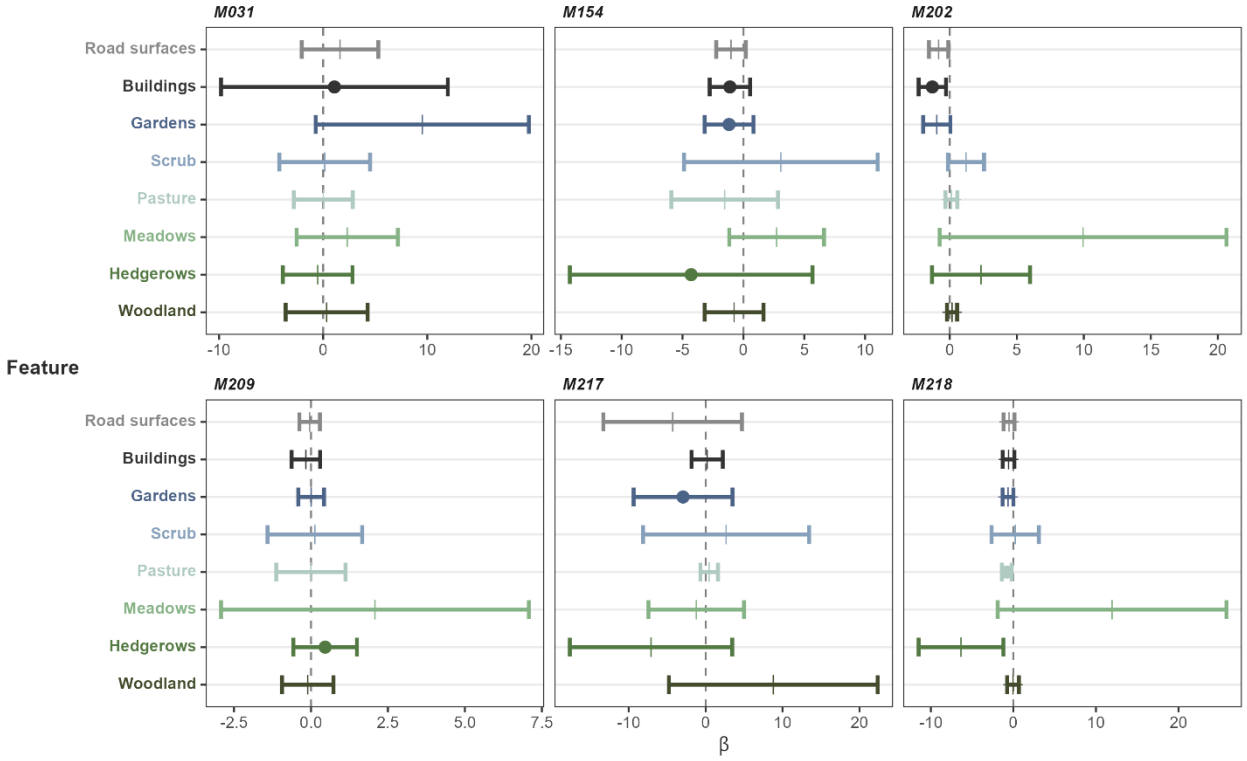

Figure 2.7: Individual integrated step selection functions demonstrating the interaction between step length and habitat features for Aesculapian snakes under the five times daily tracking regime. Positive values indicate longer steps associated with a habitat feature, and negative values indicate shorter steps. Error bars indicate 95% confidence intervals. Circles indicate the features which were included in models with the lowest AIC score or scores less than two higher.

2.3.7 Seasonality

Female movement showed a visible peak in motion variance during the egg-laying season from July to mid August (Figure 2.8). The mean daily displacement (MDD) of females during the egg-laying season was 34.05 ± 75.45 m/day compared to 12.52 ± 32.33 m/day during the rest of the

year (Figure 2.9). Across all females there was only one day where an individual moved > 100 m outside of the egg-laying season, and females were sedentary outside of this period. We discovered eggs inside the compost heap of a residential property immediately after F177 left it on 10/08/2021. F159 made an uncharacteristically long 364m move in mid-July that we interpret as nest searching, before spending three days (13/07/2021 - 16/07/2021) inside the dung pile within the Welsh Mountain Zoo. While we could not locate her eggs despite extensive search, this may be due to the size of the pile, which is approximately 10 m across. We found a separate clutch of eggs inside this pile in 2019, confirming that it is an egg-laying site for the species. One further Aesculapian snake egg was found by Zoo staff in a pile of wood chippings 20 m from the dung heap on the Zoo site in 2021.

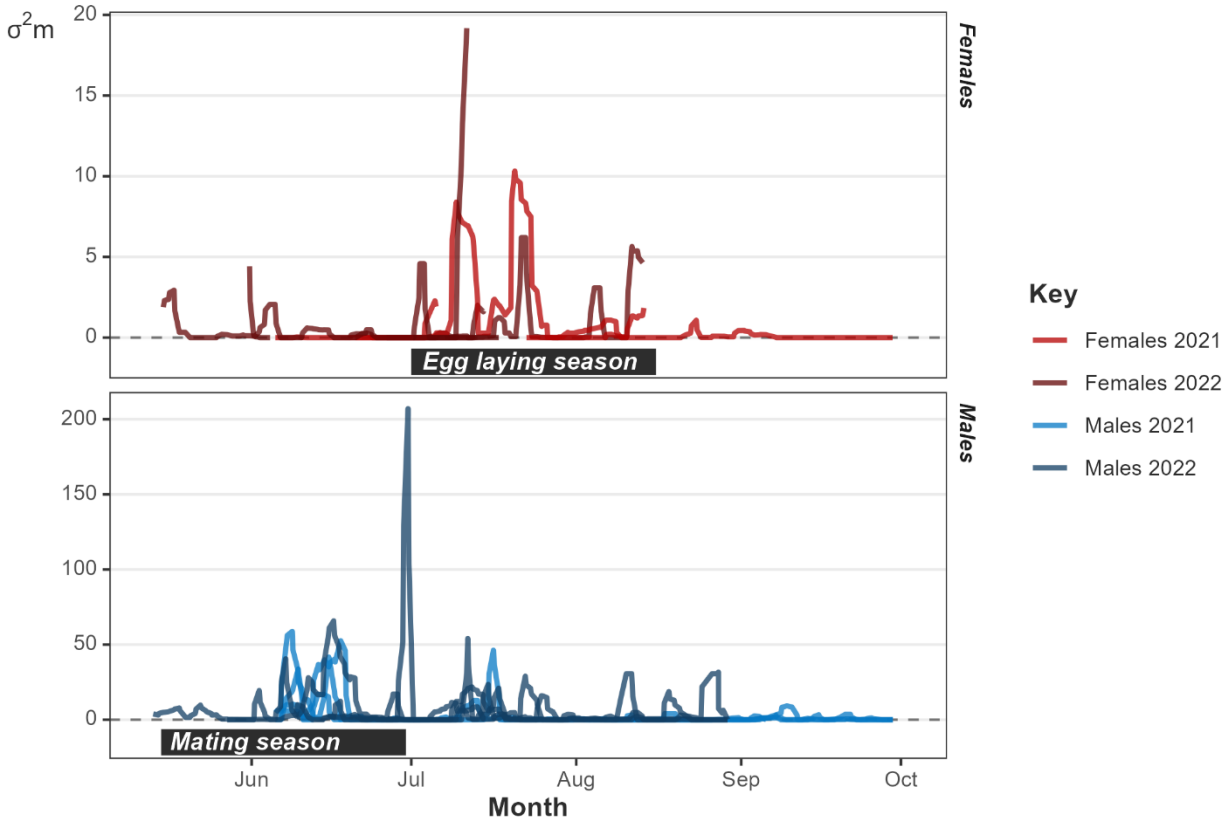

Figure 2.8: Motion variance plot for females and males through the study periods in 2021 and 2022. Peaks represent increased movement distances.

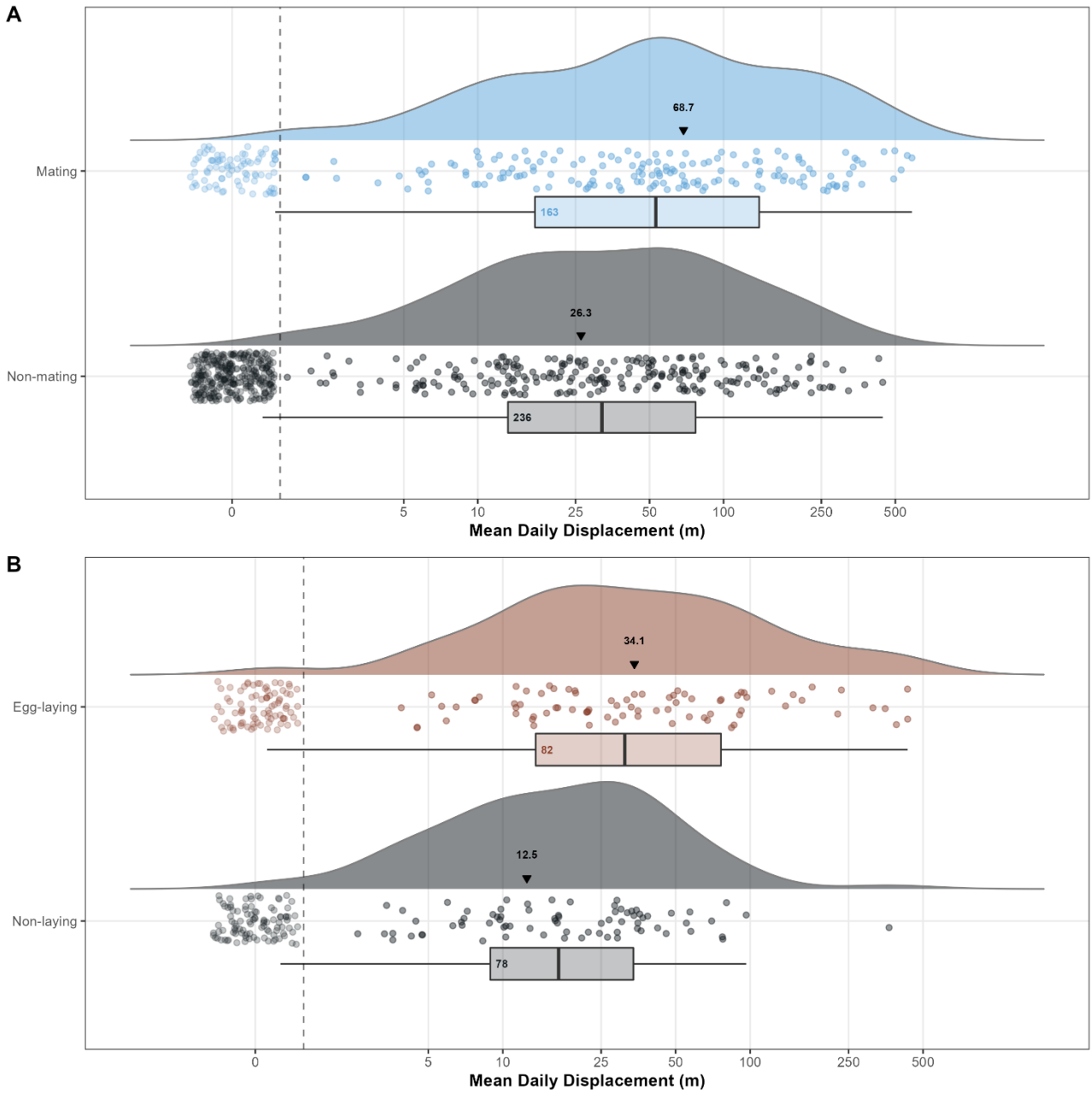

Figure 2.9: A) Raincloud plot visualising the mean daily displacement of eight tracked male Aesculapian snakes through 2021-2022 in the mating season (15th May – 30th June) and non-mating periods. B) Raincloud plot visualising the mean daily displacement of eight tracked female Aesculapian snakes through 2021-2022 in the egg-laying season (July 1st – August 16th) and the rest of the active season. For both plots, each point represents the distance moved by a snake on an individual day. Non-moves are excluded from the box and density plots, with numbers inside the box showing the points in each season. Days without movement are plotted to the far left along

with a count. Mean daily displacement for each season, with non-moves included, are printed along with a small black arrow. Figure created using code adapted from Hodges et al. (2022).

The males showed a visible peak in motion variance the mating season between mid-May and the end of June (Figure 2.8). The MDD of males during the mating season was 68.7 ± 112.22 m/day compared to 26.3 ± 58.39 m/day during the rest of the year (Figure 2.9). Males only moved distances > 500 m per day during the mating season, despite occasional periods of high activity in mid-summer. We observed one tracked male mating on 13/06/2021 and another on 24/05/2022. We witnessed one of our tracked males in combat with an unknown male on 15/06/2022. These observations reinforce our understanding of the timings of the mating season of this species in Wales.

2.3.8 Snake mortality

Of our sample of 21 tracked snakes, five died during their tracking periods, and one died after tracking was completed. In total, three females died. F177 was tracked during 2021 until the transmitter ran out of battery, and was then found dead the following year on 15/08/2022 after a car strike (Table 2.1). F142 was also killed by a car strike on 07/07/2021 after being tracked for 33 days, on the same two-laned stretch of road as F177. Both snakes were gravid at the time of death, containing four and seven well-developed eggs respectively. Lastly, F159 was cannibalised by a tracked male (M137) in August 2021 (Major et al., 2023).

Three of 13 male snakes died during the study. In 2021, M073 was predated by buzzards, with his transmitter signal discovered in a pine tree containing a buzzard nest, approximately 30 m high up. M149 died from mammal predation. The snake was found at the edge of a meadow with approximately one metre of trampled grass in all directions with a broken neck. Small sections of the snake had been consumed. We suspect either a stoat, badger, or domestic cat was responsible. Finally, in the 2022 season, M031 was killed by a car strike on the entrance road within the Welsh Mountain Zoo grounds.

2.3.9 Time spent sheltering

All snakes spent time stationary, being located at the same location for at least two tracks in succession. The mean time spent stationary of all females was 4.91 ± 4.04 days (0.5 - 14.27 days, Table 2.4). For all males, the mean time spent stationary was 5.14 ± 6.67 days (range 0.82 - 27.61

days, Table 2.4). Generally, when snakes were in the same location for multiple successive fixes, they were inside shelter. The exception were two snakes who were repeatedly found in basking sites in vegetation at the edges of roads (M137 and M218). Females selected a road verge (n = 1), compost or vegetation piles (n = 4), and buildings (n = 3) as repeated or long-term shelter. Males chose a road verge (n = 1), compost or vegetation pile (n = 1), or buildings (n = 10). Five males used two different buildings for shelter, while three males used three different buildings. These shelter sites were often used for long periods, and seven males and seven females spent ≥ 10 days in an individual shelter (Table 2.4).

Table 2.4: Time spent stationary for all 21 radio-tracked Aesculapian snakes.

ID	Mean time spent stationary (days)	Minimum time spent stationary (days)	Max time spent stationary (days)
F050	14.27 ± 1.17	0.11	20.14
F142	3.49 ± 0.32	0.03	19.47
F158	3.37 ± 0.23	0.06	11.06
F159	3.37 ± 0.3	0.02	11.44
F177	3.18 ± 0.22	0.1	18.9
F203	3.01 ± 0.12	0.04	11.28
F212	0.5 ± 0.06	0.01	1.91
F219	8.1 ± 0.78	0.005	10
M031	3.78 ± 0.53	0.04	14.6
M073	1.3 ± 0.22	0.03	2.84
M074	27.61 ± 0.81	0.09	29.02
M137	5.26 ± 0.23	0.02	15.51
M139	4.31 ± 0.37	0.02	17.01

M149	2.83 ± 0.36	0.04	8.52
M154	2.34 ± 0.17	0.02	13.73
M178	5.17 ± 0.32	0.004	18.56
M180	5.94 ± 0.52	0.01	16.1
M202	1.98 ± 0.08	0.02	9.26
M209	3.77 ± 0.24	0.02	13
M217	0.82 ± 0.06	0.02	5.1
M218	1.67 ± 0.09	0.02	7.39

2.4 Discussion

2.4.1 Anthropogenic habitat use by Aesculapian snakes

Our habitat and step selection analyses reveal that male Aesculapian snakes show a distinct preference for buildings in their introduced range in North Wales, with seven of eight individuals in our AKDE-weighted habitat selection function selecting buildings as habitat. Like many introduced species, Aesculapian snakes were introduced close to an urban area following escape from captivity. Urban areas often contain under-utilised resources, and introduced species which can capitalise on them have a significant advantage (Mitchell, Folt & Hall, 2021). For snakes, anthropogenic structures such as buildings and culverts provide shelter, thermoregulatory opportunities and egg-laying sites (Keller & Heske, 2000; Lelièvre et al., 2010; Hanslowe et al., 2016; Hodges et al., 2022; Yu et al., 2022). Aesculapian snakes are known to use anthropogenic structures as refuge in a northern part of their natural range in Czechia (Kovar et al., 2016).

Despite their ability to benefit from human features of the landscape, the semi-rural range of this introduced predator also contains dangers. Three of 21 tracked snakes died to road mortality. Road mortalities can be high for juvenile Aesculapian snakes (Kovar et al., 2014), and our study site lacks any culverts beneath the roads, which are known to be utilised by snakes for road crossing (Jones et al., 2022). Indeed, road mortality was found to be low for adult Aesculapian snakes in a population where culverts allowed safe passage under roads (Kovar et al., 2014). Two females died

on roads while heavily gravid, potentially suggesting an increased risk of road mortality when travelling to lay eggs. Reptiles are known to increase their movement distances in highly disturbed areas, despite keeping smaller home ranges (Doherty, Hays & Driscoll, 2021), further exacerbating the risk of road mortality. Three snakes demonstrated avoidance of roads in this study, suggesting they may be responsible for limiting the expansion of this species, although this is more likely the case for wider roads (Roe, Gibson & Kingsbury, 2006; Bauder et al., 2021). Certainly, the four lane A55 road to the north has presented a barrier to the species so far. Two other mortalities in this study were caused by a mammalian predator and a buzzard, and one was cannibalised by an Aesculapian snake (Major et al., 2023).

2.4.2 Habitat use and barriers to dispersal

Our AKDE-weighted habitat selection functions showed that woodland and pasture were favoured by two snakes, with one each demonstrating a preference for gardens, hedgerows, and meadows. Three males avoided roads, while one avoided woodland. While woodland is considered good habitat for Aesculapian snakes in the southern parts of their range, it may not be warm enough in more northern areas, with snakes having been shown to prefer edge habitats with better thermoregulatory potential (Kovar et al., 2016). Despite this, much of the habitat offered by the small woodland patches in our study site is likely to be edge habitat. We were unable to distinguish any habitat preference for females using the AKDE-weighted habitat selection functions, but one female showed avoidance of pasture. Our ISSF analysis showed males demonstrated habitat generalism, favouring buildings, gardens, roads, scrub, and meadows. This generalism may be due to males selecting a greater diversity of habitats when mate searching (Bauder et al., 2021). The ISSF of female snakes at the population scale showed a preference for woodland. We observed female snakes using a large tract of woodland to travel during the egg-laying season, and our step-length analysis hinted that they may travel longer distances through it. Snakes did not attempt to enter the built-up housing areas to the east and west of the site, suggesting these areas may represent barriers through which the snakes are unwilling to travel. Our data suggest that 11 of 21 tracked snakes demonstrated range residency during their tracking period. We found these individuals had home ranges of between 0.28 and 120 hectares. While previous studies have not achieved accurate estimates of home range size for Aesculapian snakes, a study of space use in France found them to use areas of 0.0067 – 5 hectares, although that study used MCPs which are likely to overestimate range size, and many snakes were tracked for short durations (Naulleau, 1989). In the present study,

males generally kept larger home ranges than females, consistent with greater activity during mate-searching. Our longest daily movement distances were longer than that of French snakes, with a maximum daily displacement for females of up to 364 m compared to 150 m. For males in our study the maximum was more than 500 m, compared to 348 m in France. Our more frequent tracking regime (twice rather than once daily) contributed to this difference.

2.4.3 Seasonality and shelter

Snakes frequently took shelter in human features of the habitat, and compost heaps, vegetation piles, and buildings represented long-term shelter for female snakes. Females spent long periods inactive during the Spring and early summer, remaining in shelter - particularly compost heaps. Males frequently used buildings as shelter sites, with one individual using a hole in a road verge and one a vegetation pile as long-term or revisited shelter sites.

Our evidence here suggests that, like other snakes, Aesculapian snakes have peaks in activity due to their reproductive cycles (Row, Blouin-Demers & Lougheed, 2012; Marshall et al., 2020). The activity of male snakes peaked in the mating season in May and June, coinciding with observations of male-male combat and mating, while females exhibited a definite peak during summer when they travel to lay their eggs. We discovered eggs in rotting vegetation built up by humans - a compost heap, a pile of wood chippings, and a dung heap at the Welsh Mountain Zoo, further demonstrating the importance of human elements of the habitat to this introduced species.

2.5 Conclusion

Despite the dangers, this study demonstrates the importance of human-dominated habitats to an introduced predator. While Aesculapian snakes are present in the fossil record of the UK, they have been absent for likely 300,000 years (Ashton et al., 1994). Worldwide, animal ranges are shifting poleward or to higher elevation as the climate warms dramatically because of human activity (Lenoir & Svenning, 2015). The UK is now home to an increasing number of mobile species which can travel over sea from further South, including numerous moths and butterflies (Sparks et al., 2007), and wetland birds (Hiley et al., 2013). Aesculapian snakes have similarly migrated northward, only via human transport instead of natural means. That said, it seems likely that North Wales represents the northernmost tolerance limit of this species in current climate conditions. The use of buildings for shelter and vegetation piles for egg laying appear to be important to their success in a temperate climate that is further north than any remaining native populations. However,

simply being successful in an area is not evidence to suggest the area contains ideal conditions (Hawley Matlaga et al., 2021), and the broad range of habitats selected by individual snakes in this study suggest that Aesculapian snakes are adaptable generalists, capable of using mixed habitat and unafraid of using buildings and other features in close proximity to humans.

Chapter 3: Aesculapian snakes show contrasting patterns of diversity across their native and introduced range

Abstract

Invasive species are recognised as one of the major threats to biodiversity worldwide, but localised introductions of non-native species receive comparatively little attention, despite making up the majority of species introductions. There is a need for monitoring of these populations to detect invasive potential, even if there are no current indications of invasiveness. Genomic approaches can tell us a great deal about these introductions. Because they have been introduced multiple times to the UK in small, isolated populations, Aesculapian snakes (*Zamenis longissimus*) represent a tractable study species to investigate this phenomenon. We explored the genetic diversity and health of two introduced populations of Aesculapian snakes in the UK, beyond their modern natural northern range limit. We produced a high quality de novo Aesculapian snake genome, and determined the population structure of the species using multiple native populations from France, Germany, Italy, and Romania. Aesculapian snakes from London, England showed a pattern of diversity consistent with heavy inbreeding and a small founding population from Italy. Conversely, the population from Colwyn Bay, Wales contained substantially higher levels of genetic diversity with an uncertain and possibly mixed ancestry. Taken together, these results show remarkably different genetic trajectories for introduced species, most likely reflecting the genetic ancestry and composition of the initial founding populations.

3.1 Introduction

Introduced species often experience rapid population growth following introduction (Kaeuffer et al., 2006; Labonne et al., 2016). In expanding populations where most individuals are reproducing, much of the genetic diversity of the founding individuals may be conserved, alleviating the effects of genetic bottlenecks (Colautti et al., 2017). This draws attention to small, established populations of non-native animals. If a population consists of relatively few individuals, and they do not undergo rapid expansion, they should be more vulnerable to extinction due to the effects of drift and inbreeding (Colautti et al., 2017). Molecular investigation of these cases is less common (Kinziger et al., 2011), but should be considered essential to our understanding of invasion biology, as the majority of established species do not spread widely (Keller et al., 2011).

The maintenance of large, connected natural populations of animals is considered crucially important to maintaining genetic diversity, and therefore the capacity for adaptation (Kardos et al., 2021). Despite this, isolated populations of introduced species originating from a small number of individuals can exhibit high levels of genetic variability, which should convey higher adaptive potential. This can stem from highly variable source populations, and multiple paternity following sperm storage by translocated females (Eales, Thorpe & Malhotra, 2008, 2010). Variation can also be maintained through admixture between organisms from multiple source populations (Kolbe et al., 2007; Dluglosch & Parker, 2008; Fuentes-Pardo & Ruzzante, 2017). In addition, isolated populations are vulnerable to inbreeding depression (Keller & Waller, 2002), putting them at greater risk of extinction (Ralls et al., 2020). Introduced species, which are usually introduced in low numbers, are particularly vulnerable (Briskie & Mackintosh, 2004). Yet in some cases, introduced species succeed without succumbing to the adverse effects of inbreeding depression, even with very small founding populations (Colautti et al., 2017).

The Aesculapian snake (*Zamenis longissimus*) is a widespread European species with a natural distribution spanning from France and north-west Spain in the west to Albania and north-west Iran at the eastern extent (Kreiner, 2007). They prey on a wide variety of prey including small mammals, birds, and lizards (Capula & Luiselli, 2002), and can grow up to two meters total length, although 1.5 m is more common, with females smaller than males (Kreiner, 2007). The species previously occupied a larger part of Northern Europe in the Holocene, only recently going extinct in Denmark during the early 1900s (Allentoft, Rasmussen & Kristensen, 2018), with remaining relict populations in Germany, Switzerland, the Czech Republic, and Poland (Musilová, Zavadil & Kotlík, 2007). There is fossil evidence of the species in south-east England dating from the middle Pleistocene 774 – 129 ka (Ashton et al., 1994; Holman, 1994; Musilová, Zavadil & Kotlík, 2007), evidencing that they reached as far north as Britain in the last interglacial period, but went extinct naturally, likely due to the intervening glacial (Holman, 1994). In modern times, the species has established two populations in the UK following human introduction (Edgar and Bird, 2006). The first, introduced in the 1970s, exists in and around the grounds of the Welsh Mountain Zoo in Colwyn Bay, North Wales. The second is in London, England, on the banks of the Regents Canal and incorporating London Zoo. This population was introduced in the 1980s.

Because of its wide distribution and multiple introductions, the Aesculapian snake offers an opportunity to study the molecular ecology of an early-stage introduced species in the context of

multiple natural populations. Previous studies have generally relied on mtDNA and nuclear microsatellite markers to determine genetic variability within introduced and conservation-relevant populations. Whole-genome sequencing represents the opportunity to use unprecedented numbers of markers, but remains under-utilised in non-model organisms, partly due to the necessity of a reference genome (Fuentes-Pardo & Ruzzante, 2017). In this study, our aims were as follows: 1) create and assemble a high-quality reference genome for the Aesculapian snake; 2) examine the population structure and genetic variability of native and introduced populations of Aesculapian snakes; and 3) determine the origin and genetic health of the two introduced UK populations.

3.2 Methods

3.2.1 Reference genome preparation

We carried out de novo assembly of the Aesculapian snake genome using linked-read sequencing. High molecular weight DNA was extracted from a female Aesculapian snake from Wales using a Qiagen MagAttract DNA extraction kit, following the manufacturer's protocol. Barcode linked-read sequencing was performed at the Centre for Genomics Research in Liverpool, using a 10x Genomics Chromium system for library preparation, and subsequent sequencing was performed using an Illumina NovaSeq. We assembled the genome using S*upernova* v 2.1.1 (Weisenfeld et al., 2017)(Weisenfeld *et al.*, 2017), using 641M reads to aim for the 56x coverage recommended by 10x Genomics, with default settings.

3.2.2 Sample locations, laboratory procedures, and genome resequencing

Samples were obtained from six countries across the natural and introduced range of Aesculapian snakes (Figure 3.1 and Table S3.1). Individuals from France, Italy, Romania, and E2 from Passau in Germany are part of the contiguous European distribution of this species. Samples A3 and B are from isolated populations in Germany. Sample B is from a relict population in Eltville, Taunus, and A3 is from an isolated population in Bretzenheim, whose origin is uncertain – it may be a modern release by humans or a historical relict population that had been overlooked until recently.

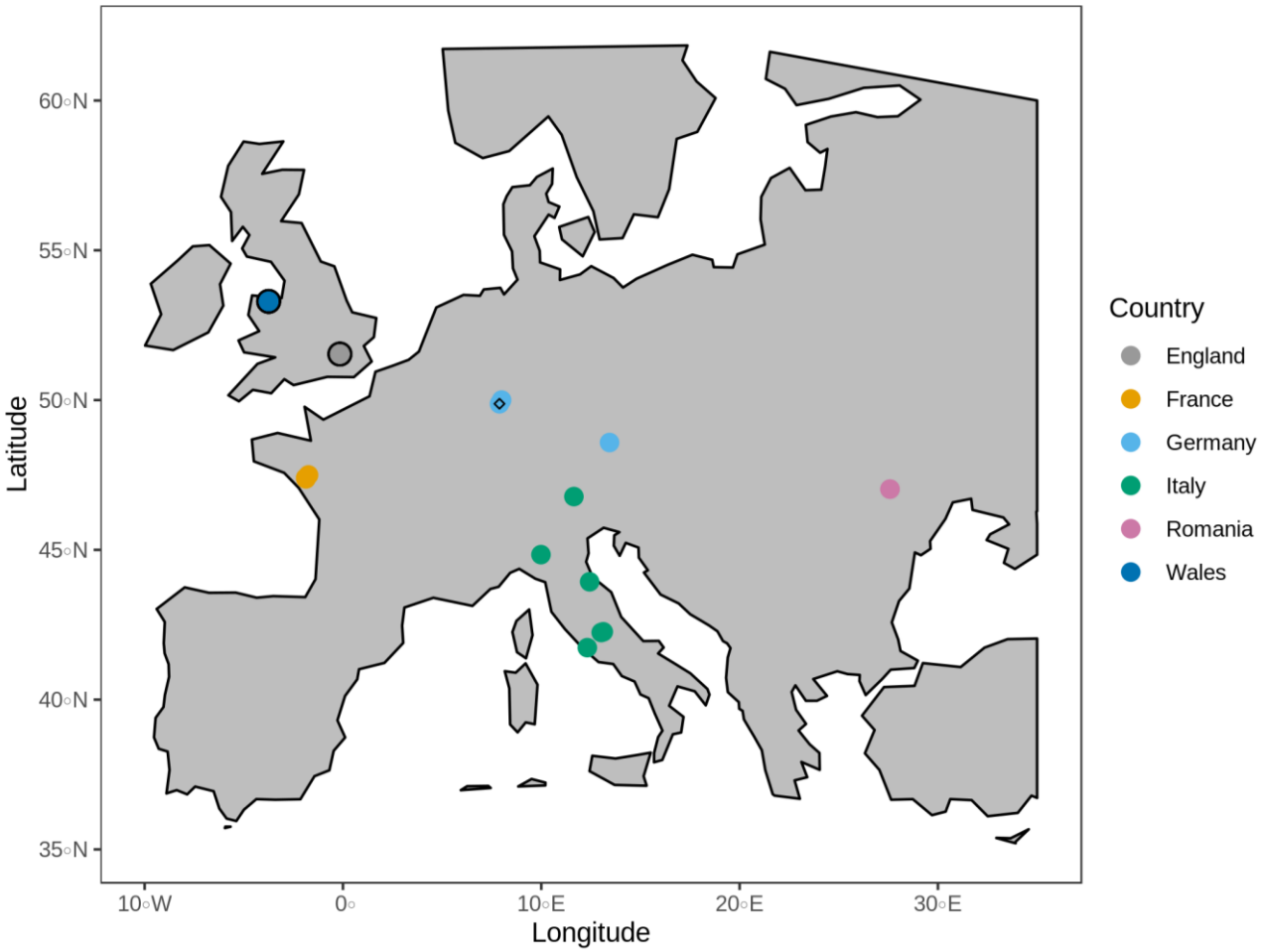

Figure 3.1: Map of Europe showing the sample locations of *Zamenis longissimus*. Samples are coloured by their country of origin according to the key. Black circles indicate known introduced populations, and the black diamond is overlaid on the population from Bretzenheim of uncertain origin, close to the native population from Eltville. The five samples from Western France are clustered closely together.

The populations in the UK are both modern introductions. Snakes from Colwyn Bay, North Wales are an introduced population found within and around the Welsh Mountain Zoo, where breeding snakes were first discovered in the 1970s. The snakes from London, England stem from an introduction in the 1980s, on the Banks of the Regents Canal. Neither population has spread substantially since their respective introductions approximately 50 and 40 years ago. Our genetic samples were a mixture of ventral scale clips and tissue samples stored in ethanol, and dry shed

skins (Table S3.1). During storage before DNA extraction shed skins were stored singly in paper envelopes. DNA was extracted using a commercial kit (Qiagen DNeasy).

Whole genome sequencing was performed by Novogene UK Ltd., Cambridge with an Illumina NovaSeq platform producing paired-end 150 bp sequencing reads. The samples were sequenced in two batches. The first included six Aesculapian snakes from Italy, six from France, six from Wales, four from London, and a captive *Zamenis situla* that was used as an outgroup for the neighbour-joining tree. Libraries were prepared by Dr. Daniel Förster at the Liebniz Institute for Zoo and Wildlife Research. These individuals were sequenced to 12 Gb data output per sample. A further three snakes from Germany and two from Romania had library preparation by Novogene and were sequenced with 20 Gb data output per sample.

3.2.3 Genomic data processing

We trimmed sequencing adapters and short reads less than 30 bp long using Cutadapt v 1.18 (Martin, 2011). Paired-end reads that were overlapping were merged using FLASH v. 1.2.11 (Magoč & Salzberg, 2011). Mapping to the reference genome was then performed using the mem algorithm of BWA v 0.7.17 (Li & Durbin, 2009). Lastly, we removed reads with poor mapping quality, potential PCR duplicates, secondary alignments, and any reads that had not mapped to our reference genome using Samtools v 1.3.1 (Danecek et al., 2021).

To allow the snake used for the reference genome to be used in our analyses, we removed index sets using Cutadapt v 1.18 by removing the first eight bases of unassembled reads. We then trimmed the pairs of fastq files, merged them and mapped them to the reference genome as described for the samples above.

3.2.4 Omitting sex chromosomes

Aesculapian snakes have genetic sex determination with a ZZ/ZW system in which females are the heterogametic sex (Pokorná & Kratochvíl, 2009). Therefore, in females, Z and W chromosomes are expected to have half the depth of coverage than that of autosomal chromosomes. In males, W chromosome coverage is expected to approximate zero. In order to exclude sex chromosomes, we analysed data from three known males and three known females. Using ANGSD (Korneliussen, Albrechtsen & Nielsen, 2014), we obtained depth (coverage) statistics (-doCounts 1 with -doDepth 1) for each scaffold. We used a minimum base quality (-minQ 30) and minimum mapping quality

(-minMapQ 30), and set a maximum depth of coverage of 100 (-maxDepth 100). Finally, we calculated the weighted mean coverage of each scaffold for each snake and visualised them (Supplementary Figure 3.1). This showed a clear pattern of reduced coverage for specific scaffolds consistent with expectations for sex chromosomes. Based on this result, we were able to assign autosomal scaffolds by applying a depth coverage cut-off of 3.5 using snake Fr4029, omitting anything below this threshold. For this and other visualisations we used R v 3.4.4 (R Development Core Team, 2012).

3.2.5 Principal component analysis

We used principal component analysis (PCA) to get an initial estimate of population structure among European Aesculapian snakes. We created a covariance matrix (-doCov 1) by randomly sampling one read (-doIBS 1) for each of our 26 Aesculapian snakes at each position of the reference genome. In ANGSD we applied these filters: for singleton exclusion we used a minimum minor allele frequency of 0.15 (-minFreq), disregarded positions that were missing data (-minInd 25), minimum base quality score (-minQ 30), minimum read mapping quality (-minMapQ 30) and we only included scaffolds over 1 Mb (-rf). PCA of the covariance matrix was computed in R using the prcomp function from Stats v 3.6.2 4 (R Development Core Team, 2012).

3.2.6 Neighbour-joining tree

We used a distance-based neighbour-joining tree to investigate phylogenetic relationships among Aesculapian snakes, using a captive *Zamenis situla* specimen as an outgroup. Using ANGSD, we first generated a distance matrix using identity-by-state distance, randomly sampling one read for each of our 26 Aesculapian snakes at each position of the reference genome (-doIBS 1). We excluded singletons using a minimum minor allele frequency of 0.15 (-minFreq), and disregarded positions with missing data (-minInd 26). Additionally, we used a minimum base quality score (-minQ30), minimum read mapping quality (-minMapQ 30), and only used autosomal scaffolds over 1 Mb (-rf). The tree was calculated and rooted in R using APE v 5.6-1 (Paradis, Claude & Strimmer, 2004).

3.2.7 Admixture

We used NGSadmix v 32 to determine genetic admixture between populations. This method is based on genotype likelihoods (Nielsen et al., 2011). We created a beagle file containing genotype likelihood calculations from all 25 Aesculapian snakes from across Europe using ANGSD (-doGlf 2). We used a SAMtools genotype likelihood model (-GL 1), estimated allele frequencies from genotype likelihoods (-doMaf 1), an SNP p-value threshold of 2e-6 (-SNP_pval 1e-6), autosomal scaffolds over 1 MB (-rf), minimum base quality score (-minQ 30), minimum mapping quality score (-minMapQ 30), omit positions with missing data (-minInd 25), minimum allele frequency (-minMaf 0.05), and set the minimum individual sequencing depth (-setMinDepthInd 2). We calculated admixture proportions with two to eight ancestral populations (K). Each of these was performed twice with different seeds (-seed).

3.2.8 Heterozygosity

To estimate genome-wide heterozygosity, we used ANGSD to estimate site allele frequency likelihoods (-doSaf 1) using our reference genome as the ancestral allele (-anc). We used the SAMtools genotype likelihood model (-GL 1), and a minimum base quality score (-minQ 30) and minimum mapQ quality (-minMapQ 30). We also set a minimum individual sequencing depth (-setMinDepthInd 3), and maximum individual sequencing depth of twice the average sequencing depth (-setMaxDepthInd). For genome-wide heterozygosity, we produced estimates of heterozygosity across 1 Mb windows using realSFS. Using ggplot2 v 3.3.3 (Wickham, 2011), we computed and drew a density plot showing heterozygous sites per kb (geom_step with stat = ‘density’).

We further explored heterozygosity rates by examining 50 kb sliding windows of the longest (103 Mb) scaffold. This allowed us to visually examine the genomes for long runs of homozygosity, a feature commonly associated with inbreeding (Keller & Waller, 2002).

3.3 Results

3.3.1 Genome assembly

Our reference genome of a female Aesculapian snake from Colwyn Bay, Wales had 53x genome sequence coverage. The assembled whole genome is 1.75 Gb, and 1.5 Gb of this is represented in scaffolds over 1 Mb (Table 3.1). We generated genome data for a further 26 Aesculapian snakes,

with genome sequence coverage ranging from 1.6x to 13x (Table S3.2). Our outgroup *Zamenis situla* sample was sequenced to 3x.

Table 3.1: Key statistics of the de novo Aesculapian snake genome assembly.

Statistic	Value
Number of reads; ideal 800M-1200M for human	641.31 M
Mean read length after trimming; ideal 140	139.50 b
Raw coverage; ideal ~56	55.33 x
Effective read coverage; ideal ~42 for raw 56x	42.65 x
Fraction of Q30 bases in read 2; ideal 75-85	90.80%
Median insert size; ideal 350-400	322.00 b
Fraction of proper read pairs; ideal >= 75	92.62%
Fraction of barcodes used; between 0 and 1	1
Estimated genome size	1.75 Gb
Genome repetitivity index	11.14%
High AT index	0.10%
GC content of assembly	41.22%
Dinucleotide content	0.12%
Weighted mean molecule size; ideal 50-100	81.83 Kb
Molecule count extending 10 kb on both sides	347.62
Mean distance between heterozygous SNPs	1.07 Kb
Fraction of reads that are not barcoded	5.07%
N50 reads per barcode	504
Fraction of reads that are duplicates	13.20%
Nonduplicate and phased reads; ideal 45-50	54.68%
Number of scaffolds >= 10 kb	8.28 K
N50 edge size	10.01 Kb
N50 contig size	30.78 Kb
N50 phase block size	1.05 Mb
N50 scaffold size	25.55 Mb

% of base assembly missing from scaffolds >= 10 kb	7.49%
Assembly size (only scaffolds >= 10 kb)	1.58 Gb

3.3.2 Population structure

Aesculapian snakes clustered according to their countries of origin along PC1 and PC2 (Figure 3.2). While distinct clusters are obvious, snakes from the population in London, England are closest to the cluster of snakes from Italy, and German and Romanian specimens are also relatively close together. Welsh specimens are well separated from any other group. Snakes from England and Romania showed the least within-population divergence, appearing closely knit on the PCA. In the neighbour joining tree (Figure 3.3), each population formed its own clade, with the exception of snakes from Italy, within which the introduced population from London were nested. Together, snakes from Italy and London formed a clade with animals from Wales. Romanian and German animals formed a sub-clade within a larger clade containing French specimens.

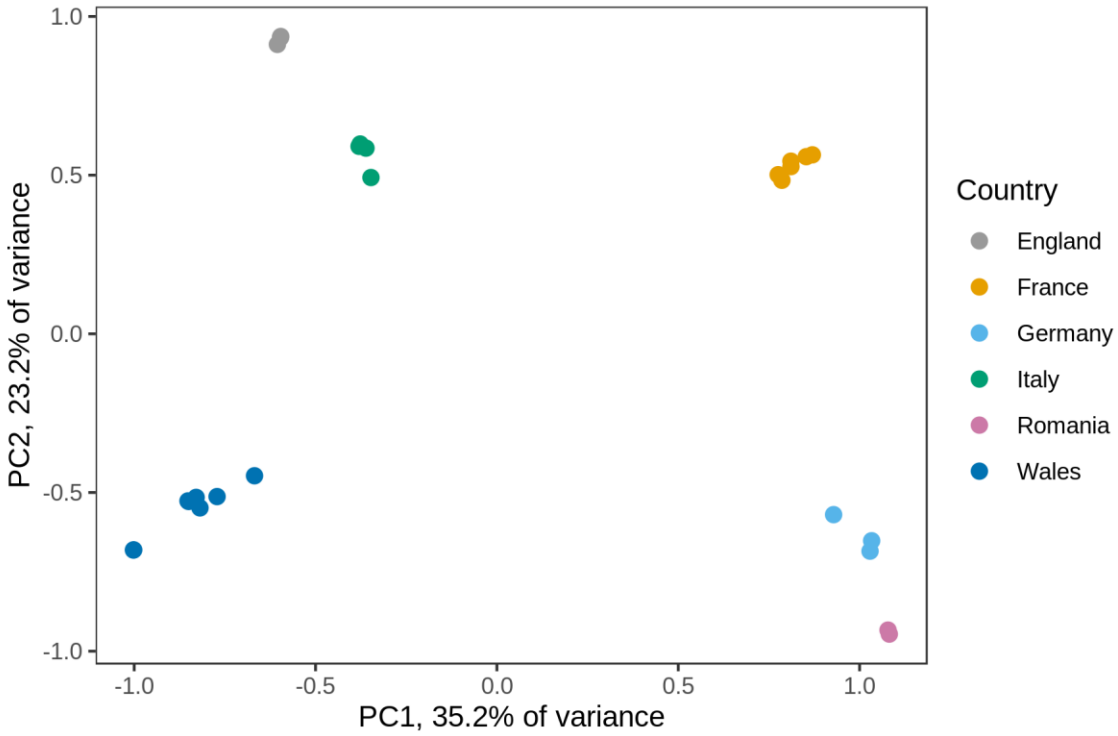

Figure 3.2: Ordination of Aesculapian snakes along the first two principal components of the PCA of genomic data, performed on over 5.6 million variable sites.

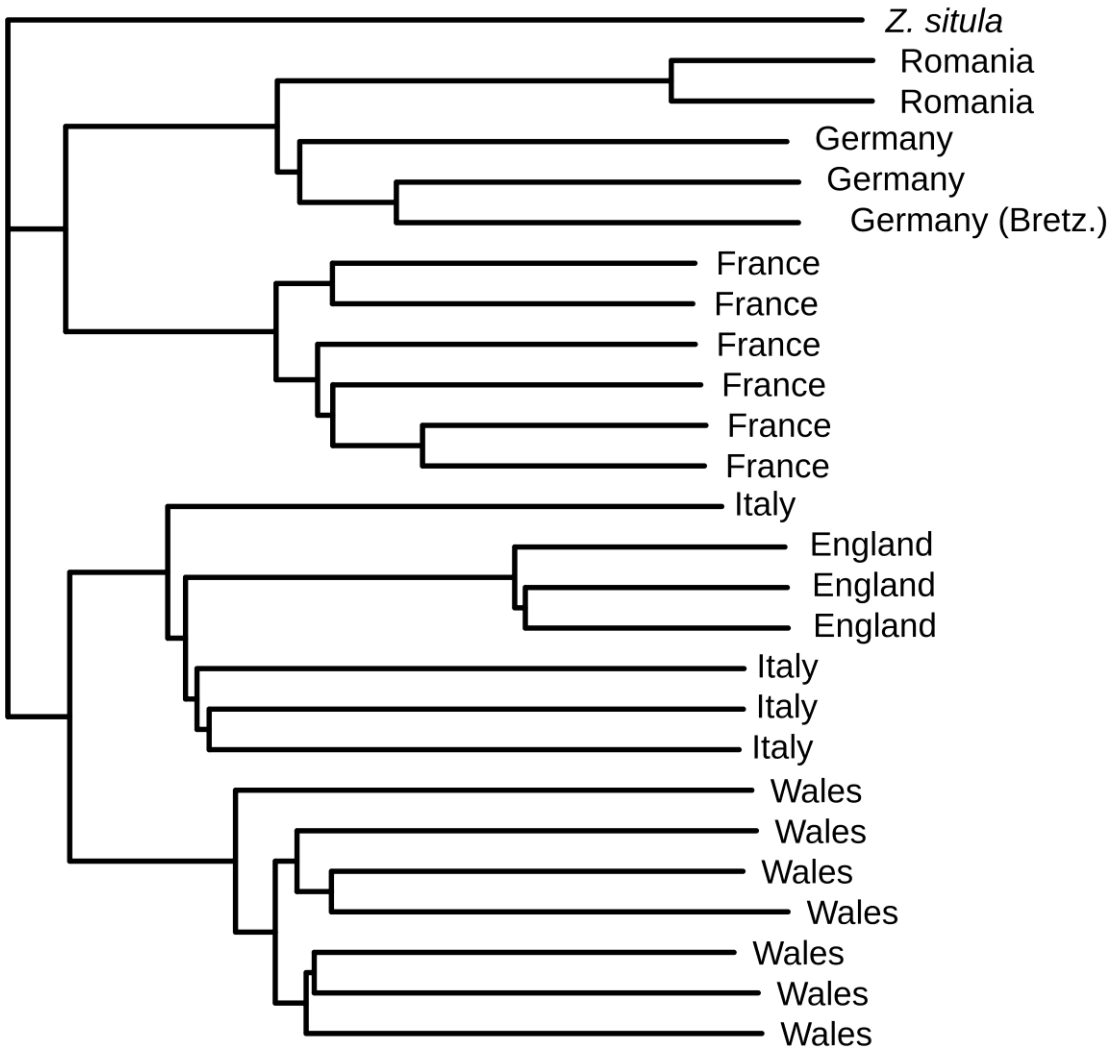

Figure 3.3: Neighbour-joining tree of Aesculapian snakes, created using whole-genome data and incorporating 5.6 million variable sites. The tree was rooted using *Zamenis situla* as an outgroup. Romania-Germany, France, and Wales are represented by four distinct lineages, with snakes from the introduced population in London nested within snakes from Italy.

The admixture population analysis broadly grouped individuals into geographic populations, and at K = 4, samples from Romania and Germany formed an Eastern grouping, with individuals from France, Wales and England having their own respective groups (Figure 3.4). At K = 4, Italy showed admixture with the populations from England, Wales, and France. Italy and France sharing a

Western grouping suggests an East-West divide in the native European populations. The assignment of individuals to populations became less consistent at K = 6 and over, with variance likely caused by inadequate resolution in the data.

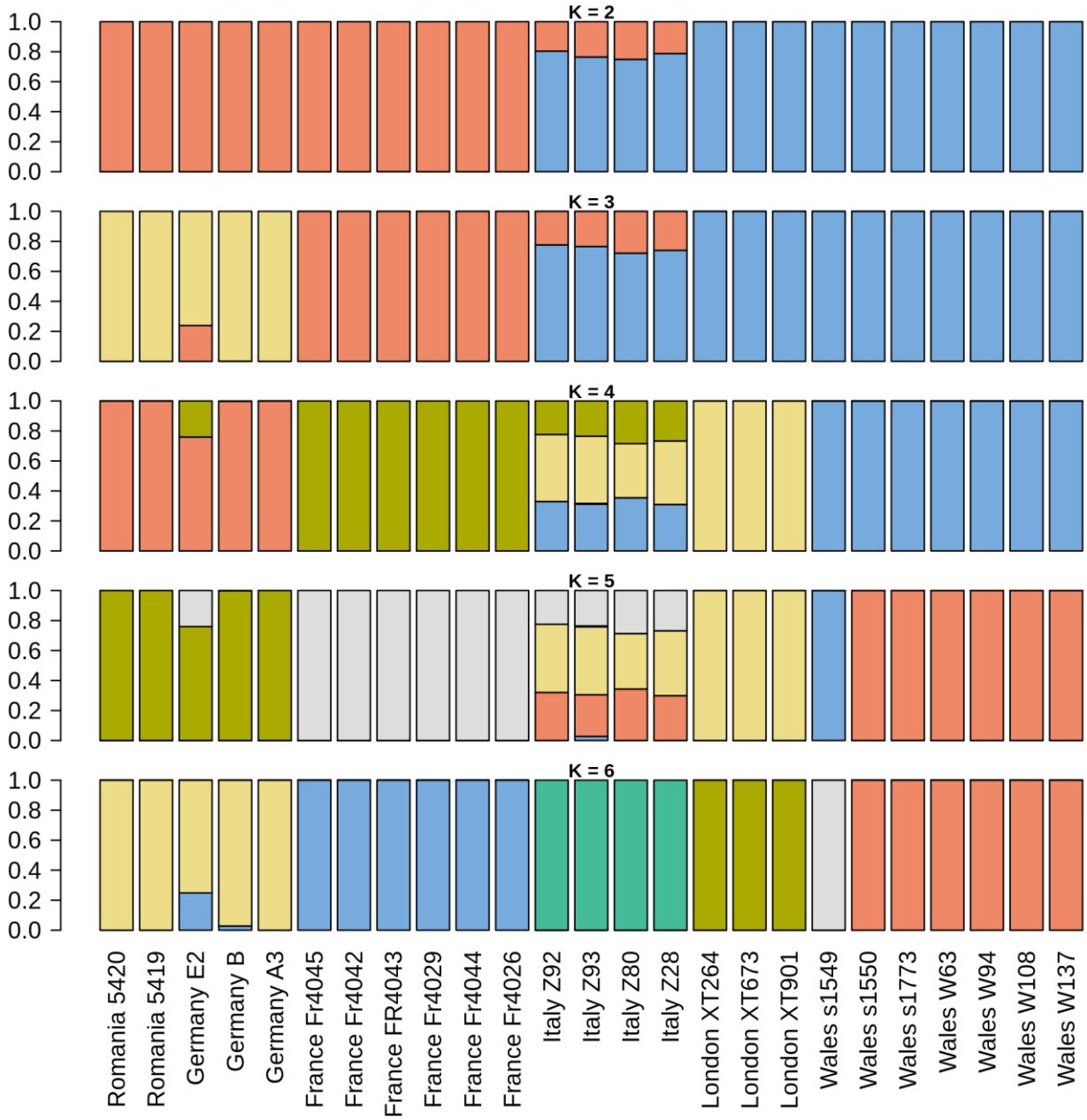

Figure 3.4: Results of the population structure analysis performed with NGSAdmix. Colours represent the different ancestral populations, the number of which is denoted by K.

3.3.3 Population genetic diversity

All Aesculapian snakes displayed a high proportion of 1 Mb windows with zero or close to zero heterozygous sites per kb (Figure 3.5). Across all individuals from all populations, median genome-wide heterozygosity ranged from 0.29 to 1.20 sites per kb, and mean genome-wide heterozygosity from 0.42 to 1.38 (Figure 3.6). Among populations, the lowest median population heterozygosity was 0.56 in London, and the highest was Italy at 1.03. The remaining median values for population

heterozygosity per kb were 0.6 for France, 0.62 for Romania, 0.81 for Germany, and 0.99 for Wales. Mean values were similar with England, France, Romania, Germany, Wales, Italy having respective means of 0.69, 0.75, 0.8, 0.96, 1.06, and 1.28.

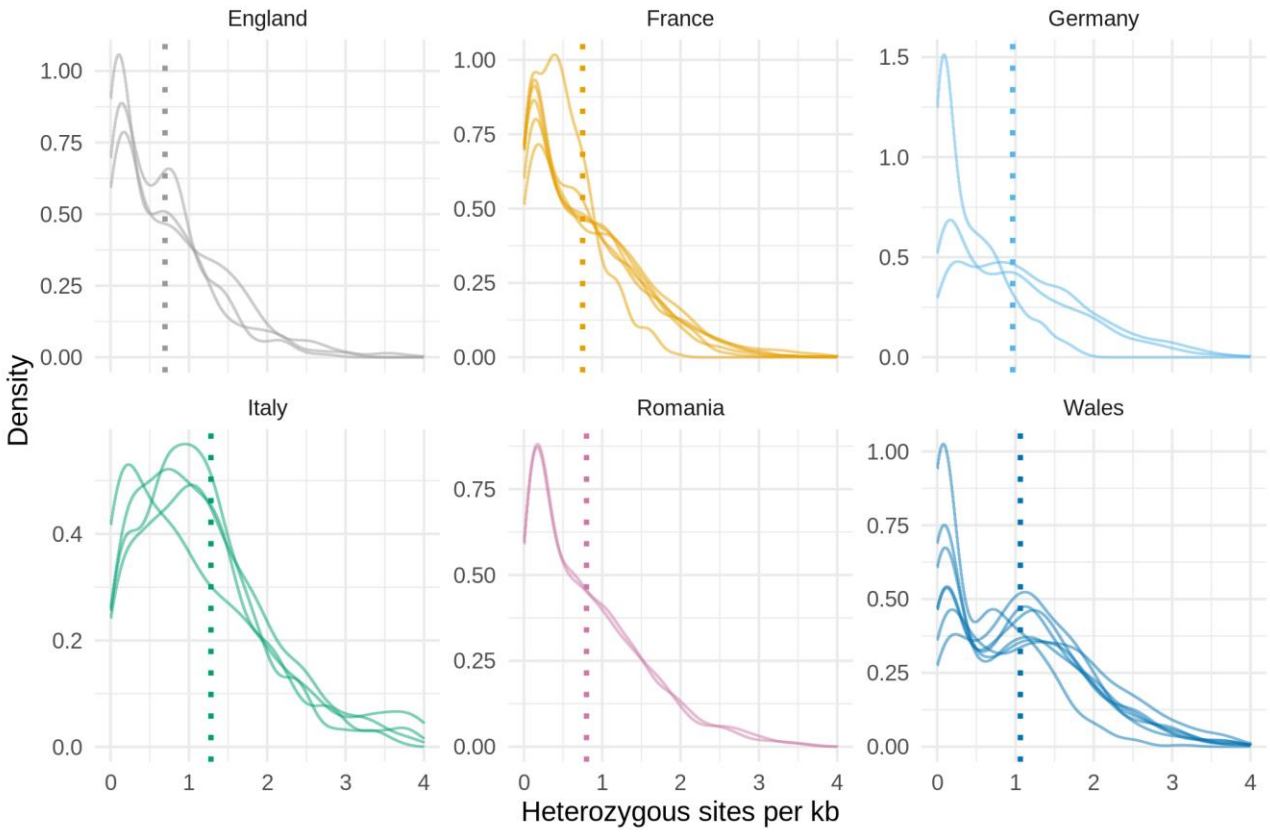

Figure 3.5: Density plot showing the genomic distribution of heterozygosity for Aesculapian snakes across 1 Mb genomic windows. Each line represents the values for one individual snake and the dashed line shows the mean heterozygosity of 1 Mb windows across all individuals of that country.

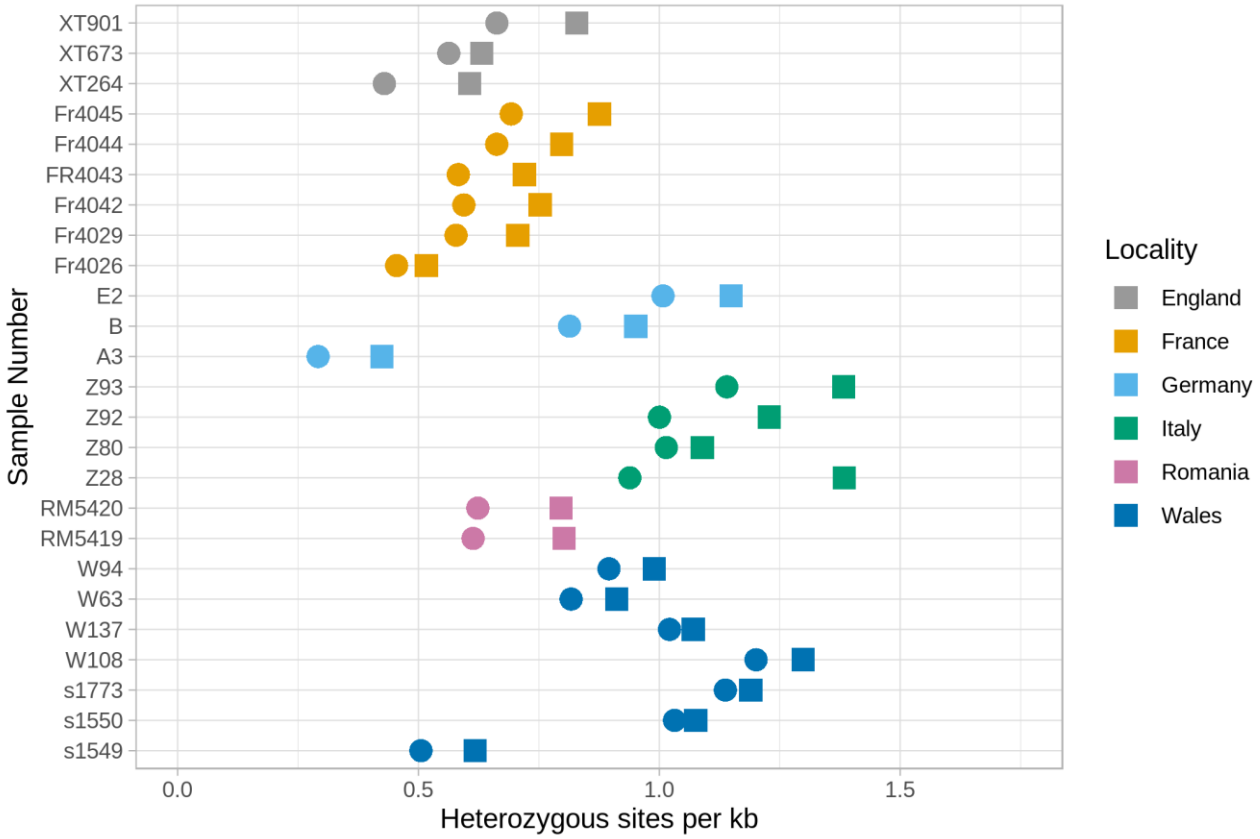

Figure 3.6: Mean and median heterozygous sites per kb across 1 Mb windows of each Aesculapian snake genome. Circles indicate median values and squares denote means.

We identified and visualised runs of homozygosity in the genomes of individual snakes using a sliding window size of 50 kb across the single largest genomic scaffold of 103 Mb (Figure 3.7). This enabled us to visualise runs of homozygosity indicative of inbreeding.

Figure 3.7: Heterozygosity across the single largest genomic scaffold in 50 kb windows for each individual Aesculapian snake in this study. Values of zero indicate homozygosity for one 50 kb window. The plots differ in length because of missing data windows, causing lower coverage datasets to produce shorter plots. Due to variable patterns of missing data, regions cannot be compared between individuals.

3.4 Discussion

Our whole genome sequencing provides insight into the population structure, genetic diversity, and inbreeding patterns of multiple natural and introduced populations of Aesculapian snakes. We determined surprisingly high levels of genetic diversity within the introduced Welsh population, as well as within the French, Italian and German samples. We revealed evidence of inbreeding in both introduced and native populations, and suggest an Italian origin for the introduced population in London, England.

3.4.1 Inbreeding

The population of snakes from London, England, suggests a strong influence of inbreeding, due to the presence of many homozygous 50 kb windows. They also possessed generally lower numbers of heterozygous sites, suggesting lower diversity. The other introduced population, in North Wales, also had runs of homozygosity suggesting some inbreeding, but with generally higher levels of genome-wide heterozygosity. Native populations from Romania and Italy showed some signs of inbreeding, but these were generally less pronounced, with the exception of Z28 from a population close to Rome which may be more isolated. French snakes showed generally fewer heterozygous sites, with some evidence of inbreeding. For German individuals, the sample from the contiguous range in Passau showed the least evidence of inbreeding, and the highest genetic diversity. The individual from the relict population in Eltville, Taunus, showed more sign of inbreeding, and the individual from the population in Bretzenheim showed extremely low levels of heterozygosity, including numerous long runs associated with inbreeding.

3.4.2 Biogeography of native populations

Aesculapian snakes have a history of range expansion and contraction in northern Europe during historical climate fluctuations (Musilová, Zavadil & Kotlík, 2007; Musilová et al., 2010; Allentoft, Rasmussen & Kristensen, 2018). Aesculapian snake phylogeography has largely been explained by an east-west divide stemming from expansion from two Pleistocene refugia in southeastern Europe and the southern Balkans (Musilová et al., 2010). Our data is consistent with populations from Germany and Romania representing the eastern, Balkan-origin clade, and Italian and specimens likely stemming from the western refugia, but our samples from France formed a clade with German and Romanian samples in our phylogeny. This is likely due to cytonuclear discordance, perhaps due to an early trans-alpine Italian lineage getting swamped at nuclear loci

by migrants from further East that subsequently went extinct in parts of Germany. France showed the lowest median value for genome wide heterozygosity of any population, with 0.6 sites per kb, and samples from Romania had 0.62 sites per kb. Contrastingly, the median value from Italy was much higher with 1.03 heterozygous sites per kb. This spatial distribution of diversity suggests that samples from northwestern France and Romania are close to the extent of Aesculapian snake expansion, as lower genomic diversity is associated with peripheral populations which are further from refugia (Stefanini et al., 2022).

The individual from Bretzenheim, Germany possessed low genome-wide heterozygosity (median 0.29 heterozygous sites per kb), and the fact that the majority of the 50 kb windows examined have little to no heterozygous positions suggest high levels of inbreeding in this population. This is in keeping with having been long-isolated from other populations. According to our admixture analysis, it is most closely related to other German individuals, suggesting German origin. The individual from the known relict population in Germany showed markedly more evidence of inbreeding than the sample from the contiguous southern part of the range. More sampling would demonstrate whether these patterns are consistent throughout those populations, and permit definite characterisation of the Bretzenheim population as introduced or relict.

The overall signal of low genome-wide diversity for native populations, and presence of runs of homozygosity, suggest that native Aesculapian snake populations are experiencing isolation and resulting bottlenecks. This is particularly evident for snakes from France, and the individual from close to Rome in Italy. These environments are heavily influenced by roads and agriculture, associated with reduced gene flow (Bauder et al., 2021), which may be contributing to their genetic erosion.

3.4.3 Dynamics of Aesculapian snake introductions

The two UK populations have different genetic origins and variability, with significantly lower levels of genetic diversity apparent in the London population than that of the Colwyn Bay population. In our phylogeny, samples from London nested within an Italian clade, suggesting an Italian origin for this population. This is in keeping with anecdotal reports that snakes held by the Inner London Education Authority, believed to be the source of the introduction, were Italian specimens. In keeping with this assertion, this population shows an inbreeding pattern consistent with a recent extreme bottleneck.

By contrast, the population from Colwyn Bay, north Wales, has maintained a high level of genome-wide genetic diversity, comparable to that of native populations in Italy and Germany. This is surprising, especially because anecdotal reports suggested this population may have been founded by a single female. While our results suggest that a single-female introduction is unlikely to have founded the entire population, snakes commonly exhibit multiple paternity within a single clutch (Uller & Olsson, 2008), and delayed fertilisation (Booth & Schuett, 2011). Either of these could be responsible for maintaining genetic diversity despite a small founding population. Alternatively, multiple individuals could have been introduced from a population with high levels of genetic diversity (Stepien et al., 2005), from multiple individuals from disparate areas, or multiple introduction events. Our structure analysis suggested admixture with snakes from Italy, and while they formed different clades in our neighbour-joining tree, they may contain Italian ancestry mixed with other areas. Burmese pythons (*Python bivittatus*) in Florida contain genomic influence from across the species' native range, and even the closely related Indian python (*Python molurus*), likely permitting higher adaptive potential (Hunter et al., 2018). There is also the possibility that the Aesculapian snakes in Wales stem from a source population somewhere in southern or central Europe with high genome-wide diversity. Despite the relatively high genome-wide heterozygosity (median 0.99 heterozygous bases per kb), samples from this population demonstrated significant runs of homozygosity consistent with inbreeding. This population only numbers around 70 adult individuals (unpublished data), making inbreeding highly likely.

3.5 Concluding remarks

We found markedly different genetic profiles between two introduced populations of Aesculapian snakes. Snakes from London, England, were determined to be of Italian origin and likely experienced an extreme population bottleneck, as evidenced by the long runs of homozygosity present in their genomes. Conversely, snakes from Colwyn Bay, Wales, demonstrated relatively high levels of heterozygosity, and likely came from a high-variation source population, mixed ancestry, or had more founding individuals. Examining genomic data from small, established populations of introduced animals alongside individuals from their broad natural distribution informs us about the impacts of bottlenecks on wild populations. Modern approaches are already beginning to investigate the environmental drivers of the genetic variation between populations (Wenne et al., 2020). Adding this context to the impacts of genetic bottlenecks and source

populations will serve to distinguish how modern species are adapting to both climate change and habitat alteration at the genomic level (Diz & Skibinski, 2023).

3.6 Funding Statement

We received funding to produce the genome from the NERC Biomolecular Analysis Facility (NBAF1242).

Chapter 4: Museum DNA reveals a new, potentially extinct species of rinkhals (Serpentes: Elapidae: *Hemachatus*) from the Eastern Highlands of Zimbabwe

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Published in *PLOS ONE* (2023). DOI: 10.1371/journal.pone.0291432.

Abstract

Genetic information plays a pivotal role in species recognition and delimitation, but rare or extinct animals can be difficult to obtain genetic samples from. While natural history wet collections have proven invaluable in the description of novel species, the use of these historical samples in genetic studies has been greatly impeded by DNA degradation, especially because of formalin-fixation prior to preservation. Here, we use recently developed museum genomics approaches to determine the status of an isolated population of the elapid snake genus *Hemachatus* from Zimbabwe. We used multiple digestion phases followed by single strand sequencing library construction and hybridisation capture to obtain *12S* and *16S* rDNA sequences from a poorly preserved tissue sample of this population. Phylogenetic and morphological analyses in an integrated taxonomic framework demonstrate that the Zimbabwean rinkhals population represents an old and highly distinct lineage, which we describe as a new species, *Hemachatus nyangensis* sp. nov. Our phylogenetic dating analysis is compatible with venom spitting having evolved in response to the threat posed by early hominins, although more data are required for a robust test of this hypothesis. This description demonstrates the power of museum genomics in revealing rare or even extinct species: *Hemachatus* from Zimbabwe are only known from a small area of the Eastern Highlands known for high

endemism. No living specimens have been seen since the 1980s, most likely due to dramatic land-use changes in the Eastern Highlands, suggesting that the species could be extinct. In view of its recognition as a highly distinct lineage, urgent action is required to determine whether any populations survive, and to safeguard remaining habitat.

4.1 Introduction

Natural history collections are vast repositories of biodiversity, archiving material from much of the planet's biodiversity, including species which may now be rare, difficult to find, or even recently extinct (Puillandre et al., 2012). These collections have formed the bedrock of species descriptions for over two centuries. However, historically, there has been a major impediment to the use of historical specimens in genetic studies (Burrell, Disotell & Bergey, 2015). Several taxonomic groups, including reptiles, amphibians, and fish, are stored in alcohol as wet collections. Over time, this preservation method degrades DNA through hydrolysis. Many specimens are further subjected to fixation in formalin prior to storage, which causes additional miscoding lesions and protein-DNA cross links that may render sequence information unobtainable using conventional means (Stiller et al., 2006; Straube et al., 2021). This has meant that the millions of specimens stored in the wet collections of natural history museums have remained largely inaccessible to genetic studies. Fortunately, the fast-evolving field of ancient DNA offers an opportunity to geneticists working with these historical samples. Ancient DNA approaches apply specialised extraction and sequencing library preparation protocols, in some cases combined with target enrichment procedures (Dabney et al., 2013; Straube et al., 2021), to successfully obtain DNA sequences from heavily degraded samples exceeding a million years in age (van der Valk et al., 2021). These same techniques can be co-opted for use in similarly-degraded samples from natural history collections (Straube et al., 2021; Bernstein & Ruane, 2022). These methods for obtaining genetic sequences from ancient and historical DNA are thus emerging as useful tools to open up the vast repositories of biodiversity to genetic analyses, including species delimitation (Jin et al., 2020). Here, we apply these techniques to investigate the status of a poorly known and possibly extinct population of the elapid snake genus *Hemachatus* from the Eastern Highlands of Zimbabwe.

Closely related to true cobras of the genus *Naja*, the African elapid snake genus *Hemachatus (*Fleming, 1822) (Fleming, 1822) currently contains a single species, *Hemachatus haemachatus* (Bonnaterre, 1790) (Bonnaterre, 1790), known colloquially as the rinkhals (Broadley, 1983; Bates

et al., 2014). These snakes are famous for displaying a defensive hooding posture when threatened, and spitting venom towards aggressors (Kazandjian et al., 2021). They are unique among Afro-Eurasian elapids in being viviparous. The genus has a southern African distribution encompassing parts of the Republic of South Africa, Lesotho, Eswatini and eastern Zimbabwe (Broadley, 1961, 1983).

The population in Zimbabwe has only been known to science since around 1920. A small, banded, and hooding snake described as a cobra was first reported from Nyanga in Zimbabwe's Eastern Highlands by a Mr J.W. Barnes, a forestry officer on what was then Rhodes Inyanga Estates (Broadley, 1961). However, the species evaded positive identification, with no complete specimens available for study until 1961, when they were identified as *H. haemachatus* by Broadley (Broadley, 1961, 1962b). While a number of further specimens were collected until the 1980s, the continued survival of *Hemachatus* in Zimbabwe is now in doubt as a result of habitat alterations: the species was last seen in the country in 1988, and more recent expeditions have failed to find evidence of its continued existence (Broadley & Blaylock, 2013). Our genetic sample was taken from an individual that was collected by a Mr G. Puttent at the Nyanga Trout Hatchery in 1982. The specimen was given to one of us (D.G. Broadley) at the Natural History Museum of Zimbabwe (NMZB - 9503) and subsequently examined. The specimen had been preserved and handed to the museum in a jar filled with some form of alcohol and labelled as such. Whether or not the specimen was ever exposed to formalin is unknown to us.

The available evidence suggests that the Zimbabwean rinkhals population was - or maybe still is - restricted to the Eastern Highlands of Zimbabwe. The Eastern Highlands are a centre of endemism and biodiversity, containing nine endemic animal species including a crab (Phiri & Daniels, 2013), a gecko (Broadley, 1962a), and a skink (Hewitt, 1932). The rinkhals population in Zimbabwe is separated from the nearest southern localities in northeastern Mpumalanga Province, South Africa, by approximately 700 km. This separation, in conjunction with the status of the Eastern Highlands as a centre of endemism, led us to the hypothesis that this population could potentially represent an unrecognised taxon, distinct from *H. haemachatus*. Its apparently small range and recent decline (against a background of a global assessment of *H. haemachatus* as Least Concern (Bates et al., 2014; Alexander, 2022)) highlights the need to establish its taxonomic status to allow an assessment of its conservation status and potential recovery action (May, 1990; Morrison et al., 2009).

Here, we adopt an integrative taxonomic approach to determining the status of the rinkhals in Zimbabwe. Based on its geographical isolation, we hypothesise that the Zimbabwean rinkhals is likely to represent a distinct evolutionary lineage. In accordance with Padial et al. (Padial et al., 2010), we then assess its status by testing for evidence of temporal lineage separation using mtDNA sequence divergence to identify the rinkhals in Zimbabwe as a candidate species. In view of the limitations of mtDNA-based inferences, we then test for congruent phenotypic variation through univariate morphometric analysis to establish the different lineages as Confirmed Candidate Species (Padial et al., 2010).

4.2 Materials and Methods

4.2.1 Historical specimen DNA extraction and library preparation

A 50 mg piece of liver tissue was removed from the historical specimen (NMZB 9503) and used for DNA extraction and Illumina sequencing library preparation. The procedures followed those described in (Straube et al., 2021), and were carried out using appropriate measures to avoid cross-contamination (i.e. clean room facilities, use of negative controls). Initially, the historical sample was subjected to a non-destructive digestion in guanidinium thiocyanate buffer ("Guanidine treatment" (Straube et al., 2021)) followed by DNA purification using the method of (Dabney et al., 2013). The undigested tissue pellet was then re-digested using a Proteinase K buffer ("Proteinase K re-digestion treatment" (Straube et al., 2021)) and the same method of DNA purification applied. The purified DNA extracts were then quantified using a Qubit fluorometer with high sensitivity reagents. Dual indexed sequencing libraries were prepared from 12 ng of each DNA extract using a single stranded method (Gansauge & Meyer, 2013) with the modifications described in (Straube et al., 2021). Uracil DNA-Glycosylase and Endonuclease VIII were used to excise uracil residues, which can occur in historical DNA molecules as a result of cytosine deamination. The optimal number of indexing amplification cycles was determined in advance using qPCR analysis of the unamplified library (Straube et al., 2021). Amplified libraries were quantified using a Qubit fluorometer with high sensitivity reagents and an Agilent TapeStation instrument with D1000 reagents. Shotgun sequencing was performed using an Illumina NextSeq 500 Instrument producing 75 bp single-end reads (Paijmans et al., 2017).

4.2.2 Assessment of endogenous DNA content

We interrogated the shotgun data from the historical sample for the presence of rinkhals DNA. Data processing was carried out using the publicly available BEARCAVE scripts (https://github.com/nikolasbasler/BEARCAVE). Briefly, adapter sequences were trimmed using the software CutAdapt (Martin, 2011), requiring a single base overlap. Reads < 30 bp after trimming were discarded. The trimmed reads were then mapped to the phylogenetically closest reference nuclear genome sequence available, that of the king cobra (*Ophiophagus hannah*) (Vonk et al., 2013) using the aln algorithm of the software bwa, with subsequent filtering for mapping quality (-Q 30) and removal of potential PCR duplicates (rmdup) using samtools (Li et al., 2009). We also mapped reads to the mitochondrial genome of the Chinese cobra (*Naja atra*; GenBank accession: EU913475), using a range of allowed mismatches in bwa (-n 0.04, 0.01, 0.001). None of these analyses were able to recover a substantial number of mapped reads, leading us to conclude that the proportion of endogenous rinkhals DNA molecules in DNA extracted from the historical sample was extremely low. We therefore chose to target the mitochondrial genome of the historical specimen for recovery using hybridisation capture.

4.2.3 Draft assembly of a modern rinkhals mitochondrial genome

Hybridisation capture requires nucleotide “baits" with high sequence similarity to the target region(s). These can take the form of PCR products which can be converted into baits for hybridisation capture (González Fortes & Paijmans, 2019). However, to our knowledge, no suitable primers exist for the amplification of the complete mitochondrial genome of the rinkhals or any close relatives. To overcome this, we extracted DNA from a ventral scale tissue sample from a modern rinkhals specimen and carried out shotgun sequencing. DNA was extracted using a commercial kit (Qiagen DNeasy) and a dual-indexed Illumina sequencing library prepared using the double-stranded method described in (Meyer & Kircher, 2010), with the modifications described in (Henneberger, Barlow & Paijmans, 2019). Sequencing and adapter trimming of the data were carried out as described above. We then generated a draft assembly of the mitochondrial genome sequence of the modern specimen using an iterative mapping method with the program MITObim (Hahn, Bachmann & Chevreux, 2013), using the Chinese cobra mitochondrial genome as the initial bait sequence and default parameters. The resulting draft assembly was imperfect with

several large sequence gaps. However, it was sufficient for the design of PCR primers to enable the amplification of the modern rinkhals mitochondrial genome using long-range PCR.

4.2.4 Long-range PCR primer design, bait preparation, and hybridisation capture

From the draft modern rinkhals mitochondrial genome sequence, we designed two sets of primers to amplify the mitochondrial genome in two overlapping sections using Primer3Plus (Untergasser et al., 2007), selecting a preferred primer length of 18–22 bp and a maximum product size of 10 kb. Specificity of the designed primer sets were then checked by BLASTn analysis against the NCBI nucleotide database. Long-range PCR was carried out using these primers and the DNA extract of the modern rinkhals as template, following the procedure described in (González Fortes & Paijmans, 2019). Hybridisation capture baits were then prepared by pooling the PCR products in an equimolar ratio, shearing using a Covaris S220 focused ultrasonicator, after which biotinylated adapters were ligated following the procedures described in (González Fortes & Paijmans, 2019).

Using these baits, we performed two rounds of hybridisation capture on the historical Zimbabwean rinkhals sample libraries, with library application cycles selected using qPCR analysis after each capture round (González Fortes & Paijmans, 2019). We also prepared an amplicon sequencing library from the modern rinkhals sheared long-range PCR products using the double-stranded method described above (Paijmans et al., 2017). Libraries were sequenced as described above.

4.2.5 Reconstruction of the historical mitochondrial sequence

We used the amplicon data from the modern rinkhals to generate an improved rinkhals mitochondrial reference sequence for mapping. This was achieved using a custom iterative mapping procedure that involved mapping of the amplicon data to the Chinese cobra mitochondrial reference using the bwa mem algorithm, and the removal of unmapped reads and non-primary alignments using samtools. A consensus sequence was then called in Geneious v7 using a 50% majority rule, retaining the reference sequence for regions lacking mapped reads. This procedure was repeated ten times, each time using the newly generated consensus sequence as mapping reference, at which point no new reads were mapped. The consensus sequence generated in the final iteration used a 90% majority rule, required a minimum depth of 3 reads, and did not retain the reference sequence for regions lacking mapped reads. The original reads were then re-mapped

using the more stringent bwa aln algorithm using this consensus as reference sequence, and a final consensus generated from this alignment in Geneious using an 85% majority rule and a minimum depth of 10 reads.

We used this improved rinkhals mitochondrial reference sequence to map both shotgun and hybridisation capture data from the historical specimen, using bwa aln with default mismatch parameter and the filtering methods described in "Assessment of endogenous DNA content". The aligned data from the historical specimen was then viewed using samtools tview. Being a small and manageable mitogenome, the coverage was evaluated by eye to determine which regions of the mitogenome we could be most confident in. Two regions with a coverage of three or more reads were found which roughly corresponded to regions of the *12S* ribosomal RNA gene (563 bp) and *16S* ribosomal RNA gene (621 bp).

4.2.6 mtDNA sequencing of additional modern specimens

We collected seven tissue samples of modern rinkhals (*H. haemachatus*) from across its range in southern Africa (Figure 4.1). Samples were collected under Cape Nature permit no. AAA004-00127-0035 or from captive specimens of known provenance, and this work took place with approval from the Bangor University Animal Welfare and Ethics Review Board. DNA was extracted as described for the modern sample above and quantified using a Nanodrop spectrometer. We then PCR amplified and Sanger sequenced the mitochondrial *12s* and *16s* genes, overlapping the recovered regions for our historical specimen. Primers and relevant target genes are presented in Table 4.1. PCR was carried out in 15 µl volumes using GoTaq® Green Master Mix and 1-3 µl of DNA template. Thermocycling programmes are provided in Tables S4.1 and S4.2. Following PCR, each sample was prepared for sequencing with the addition of 1 µl exonuclease 1 (20 U/µl) and 2 µl of thermostable shrimp alkaline phosphatase (1 U/µl), followed by a 15 minute incubation at 37 °C and an enzyme inactivation period of 15 minutes at 85 °C. The *12S* fragments were sequenced in both directions and we created a consensus of the two sequences using Geneious version 2020.1 (Kearse et al., 2012).

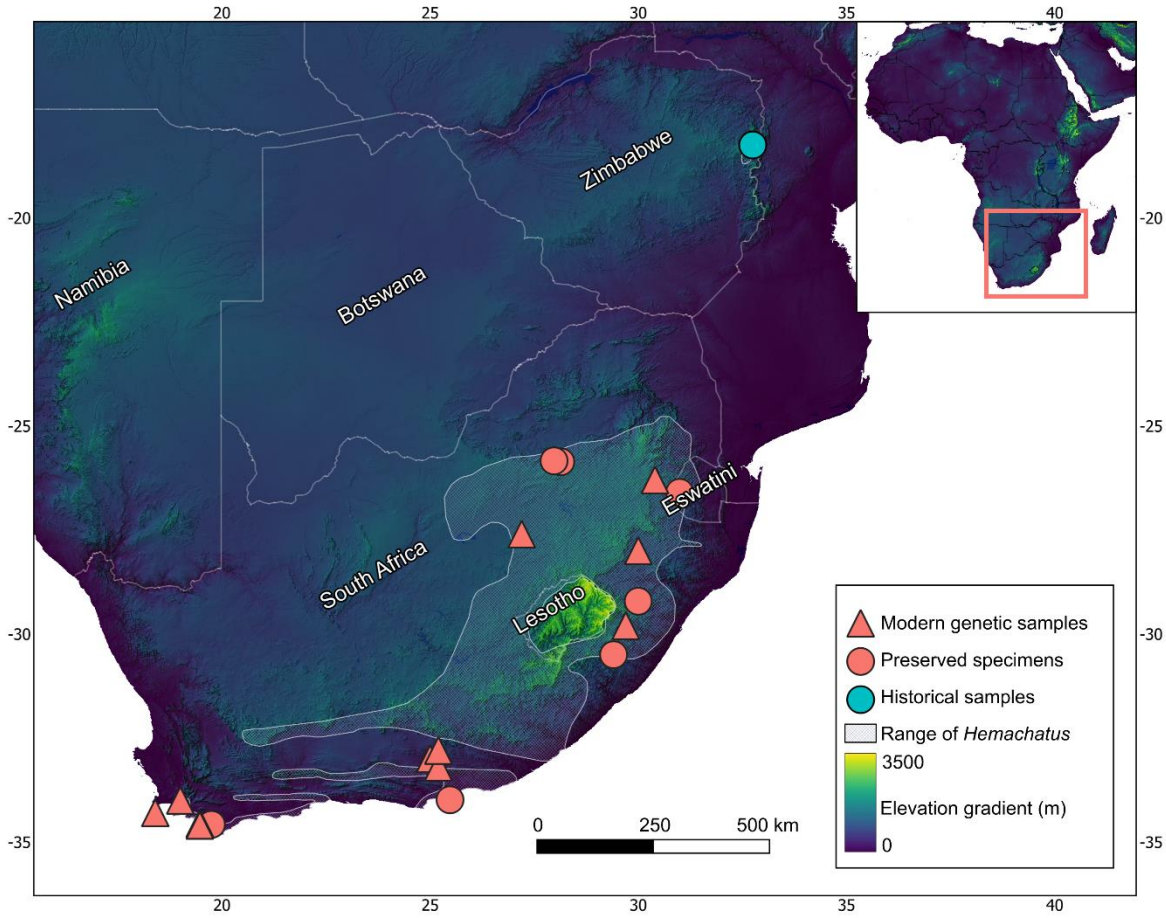

Figure 4.1: Localities for historical and modern rinkhals included in the analyses. Ten individuals from South Africa included in the morphological analysis were excluded from the map as their locality was only recorded to province level (S4.4 Table). Preserved specimens from South Africa are those used in the morphological analysis. Overlaid locations have been adjusted to improve visibility. Public domain elevation data were sourced from the USGS Earth Resources Observatory and Science Center (Earth Resources Observation And Science (EROS) Center, 2017). Range layer for *Hemachatus* reprinted from (Alexander, 2022) under a CC BY license, with permission from the IUCN, original copyright 2022.

Table 4.1: Primers used in the PCR process.

Genes	Primers ('5 to 3')	Reference
12S	*12S* rRNA, AAAGTATAGCACTGAAA	(Kocher et al., 1989)
	tRNA-Val, GTCGTGTGCTTTAGTCT	(Kocher et al., 1989)
16S	L2510, CGCCTGTTTATCAAAAACAT	(Palumbi et al., 1991)
	H3059, CCGGTCTGAACTCAGATCACGT	(Palumbi, 1996)

4.2.7 Phylogenetic analysis and molecular dating

To investigate the timing of the divergence between Zimbabwean and southern African populations of *Hemachatus*, we aligned our *16S* and *12S* sequences with corresponding sequences from other species of the genera *Naja*, *Pseudohaje*, *Walterinnesia*, *Aspidelaps* and, as outgroup, *Ophiophagus*, taken from GenBank and our own unpublished data. To increase the overall robustness of the tree, we concatenated this alignment with additional sequences of the mitochondrial genes for cytochrome *b* (cyt-*b*) and NADH dehydrogenase subunit 4 (ND4) from (Kazandjian et al., 2021) for all taxa except the Zimbabwe *Hemachatus*. All sequences were aligned in MEGA 11 (Tamura, Stecher & Kumar, 2021) using the MUSCLE alignment algorithm and default settings (Edgar, 2004). In both *12S* and *16S*, a short region of hypervariable sequence was excluded from further analysis.

Phylogenetic reconstruction and molecular dating were carried out in BEAST v. 1.10.4 (Suchard et al., 2018). To calibrate the tree, we constrained several highly supported key nodes according to the multilocus phylogeny of Kazandjian et al. (Kazandjian et al., 2021), and constrained and applied normal priors to their date. The mean date obtained by the previous molecular dating study

(Kazandjian et al., 2021) was set as the mean, with a standard deviation of 0.01, effectively fixing the node date. The constrained nodes and their dating were the cobra clade *Aspidelaps* + *Walterinnesia* + *Hemachatus* + *Pseudohaje* + *Naja* (19.7 Mya), *Hemachatus* + *Naja* + *Pseudohaje* (17.0 Mya), and the subgenera *Afronaja* (6.74 Mya), *Naja* (5.44 Mya) and *Uraeus* (3.71 Mya). A lognormal uncorrelated relaxed clock model was used. The optimum data partitioning scheme was identified using Partitionfinder v. 1.1.1 (Lanfear et al., 2012), using the greedy search algorithm in conjunction with PhyML (Guindon et al., 2010). The identified partitions were *12S*, *16S*, and the three codon positions of the combined, concatenated protein-coding genes, cytochrome b (*cyt-b*) and NADH dehydrogenase 4 (*ND4*). We implemented the general time-reversible model with four gamma categories, empirical base frequencies, gamma shape parameter and proportion of invariable sites for each partition, as recommended by Partitionfinder, with the departure that, for operational reasons, we used the GTR+I+G model for the third codon positions of the two protein-coding genes, as opposed to GTR+G, as supported by Partitionfinder. Since most nodes of the phylogeny were interspecific, we implemented a Yule Speciation Process Tree. The analysis was run for 20 million generations with a 10% burn-in period. Burn-in and the effective sample size (ESS) of all parameters were verified using Tracer v. 1.7.1 (Rambaut et al., 2018).

4.2.8 Morphological analysis

We included 22 individuals of *Hemachatus* from South Africa and 14 from Zimbabwe in the morphological analysis (Table S4.3). We recorded sex, snout-vent length (SVL), and tail length (TL). We counted the number of nape scale rows, midbody scale rows, pre-cloacal scale rows, cloacal scales, subcaudal scales, upper labials, upper labials entering orbit, lower labials, lower labials in contact with the anterior sublinguals, preocular, and postocular scales. Due to the state of preservation of the specimens, data were not recorded for every character in all snakes (Table 4.1, Table S4.4). Depending on the character, we used chi-squared, Fisher's exact, and two-way analysis of variance (ANOVA) tests to evaluate the influence of group and sex on all characters. As size can be a confounding factor in the analysis of growth-related characters, and because TL is likely to be sexually dimorphic, we performed a two-way analysis of covariance (ANCOVA) on TL data using SVL as the covariate (Packard & Boardman, 1999).

Finally, since even long-separated lineages may lack absolutely diagnostic characters, we conducted a principal component analysis (PCA) using the meristic variables that differed

significantly between the two populations in order to visualise the distinctness of the two forms in multidimensional morphospace, free of the confounding effects of a priori group assignment. This analysis included midbody scale rows, nape scale rows, number of ventral scales, and number of subcaudal scales. All characters were standardised to zero mean and unit standard deviation prior to analysis. For the PCA, we performed a singular value decomposition of the covariance on data from 12 snakes from South Africa and 11 from Zimbabwe.

4.2.9 Nomenclatural acts

This published work and the nomenclatural acts it contains have been registered in ZooBank, the online registration system for the ICZN. The ZooBank LSIDs (Life Science Identifiers) can be resolved and the associated information viewed through any standard web browser by appending the LSID to the prefix "http://zoobank.org/". The LSID for this publication is: urn:lsid:zoobank.org:pub:CB9F0F49-FCDA-4288-A693-5187A810B24C.

4.3 Results

4.3.1 Genetic analysis

We recovered 14,791 bp of unambiguous sequence for the mitogenome of the modern rinkhals sample ZRP2264. The missing parts of the mitogenome corresponded primarily to the two control regions (many snakes have two identical or near-identical mitochondrial control regions (Kumazawa et al., 1996)). Against this reference, we were able to align 349 bp of *16S* and 394 bp of *12S* unambiguous sequence of the Zimbabwean sample.

For phylogenetic and molecular dating analyses, we aligned a total of 2,311 base pairs of mitochondrial DNA (*12S*: 512 b.p.; *16S*: 483 b.p.; *cyt-b*: 657 b.p.; *ND4*: 659 b.p.) for all specimens except the Zimbabwean rinkhals, for which only partial *16S* and *12S* sequences were available. The sequences used and their GenBank accession numbers are shown in Table S4.4.

All parameters of the analysis were confirmed to have reached convergence in Tracer, with ESS values of over 500 in all cases. The maximum clade credibility tree (Figure 4.2) confirms the monophyly of *Hemachatus* with high levels of support (posterior = 0.98), and an estimated age of 10.14 Mya (95% HPD = 6.25-14.39 Mya) for the split between the Zimbabwean *Hemachatus* and

the samples from the remainder of the range. Excluding the Zimbabwe lineage, the age of first divergence within *H. haemachatus* is 0.69 Mya (95% HPD = 0.46-0.96 Mya).

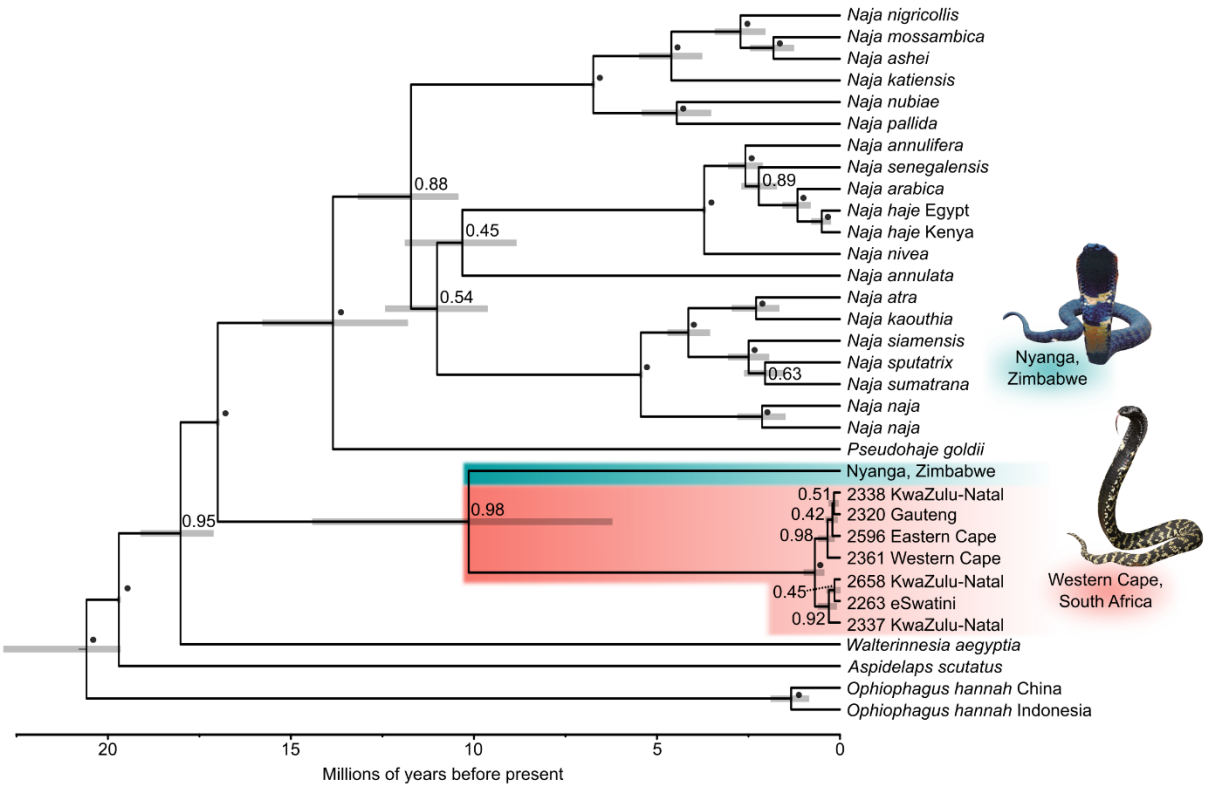

Figure 4.2: Dated Bayesian phylogenetic tree of the cobra group of elapids. Grey bars denote 95% HPD confidence intervals for divergence times; nodes lacking grey error bars were used as calibration points. Node labels represent Bayesian posterior probabilities (BPP), with black dots denoting BPP ≥ 0.95. The clade representing *Hemachatus* individuals from South Africa and Eswatini is highlighted in salmon pink, and the divergent Zimbabwe lineage in blue-green.

4.3.2 Morphology

Our univariate morphological analyses demonstrate that rinkhals from Zimbabwe possess significantly fewer midbody scale rows, nape scale rows, ventral scales, and subcaudal scales than those from South Africa (Table 4.2, Table 4.3, and Figure 4.3). Our PCA visualises the differences in overall phenotype across the four characters included, despite the absence of absolutely diagnostic single characters. Eigenvector coefficients of the characters in the PCA are presented in Table 4.4: broadly, higher counts in all meristic characters, especially subcaudals, are associated

with higher PC 1 scores, and higher ventral scale counts with high PC 2 scores. Consistent with the univariate analyses, South African specimens exhibit generally higher PC 1 scores than those from Zimbabwe (Figure 4.4), reflecting their generally higher scale counts, especially subcaudals.

Table 4.2: Results of statistical tests comparing the morphological characters of South African and Zimbabwean *Hemachatus*. ANOVA, chi-squared tests, and Fisher's exact tests were used where appropriate. In the *P* column, asterisks highlight significant differences or interactions ($P < 0.001$ ***, < 0.01 **, < 0.05 *).

Character	Test	Effect	d.f.	Value	*P*
Midbody scale rows (17 - 18, or 19)	Chi-squared test	population	1	$X^2 = 4.49$	0.034 *
Nape scale rows (16 - 18, or 19)	Fisher's exact test	population	-	-	0.02 *
Pre-cloacal scale rows (11 or 13 or 14)	Fisher's exact test	population	-	-	0.697
Supralabials (6 or 6.5 or 7)	Fisher's exact test	population	-	-	1.000
Subcaudals	Two-way ANOVA	population	1	F = 25.839	< 0.001***
Subcaudals	Two-way ANOVA	sex	1	F = 4.009	0.055
Subcaudals	Two-way ANOVA	population * sex	1	F = 0.133	0.718
Ventrals	Two-way ANOVA	population	1	F = 9.036	0.006 **
Ventrals	Two-way ANOVA	sex	1	F = 16.935	< 0.001 ***

Ventrals	Two-way ANOVA	population * sex	1	F = 1.822	0.189
Tail length	Two-way ANCOVA	population	1	F = 2.659	0.125
Tail length	Two-way ANCOVA	sex	1	F = 14.547	0.002 *
Tail length	Two-way ANCOVA	population * sex	1	F = 1.910	0.189

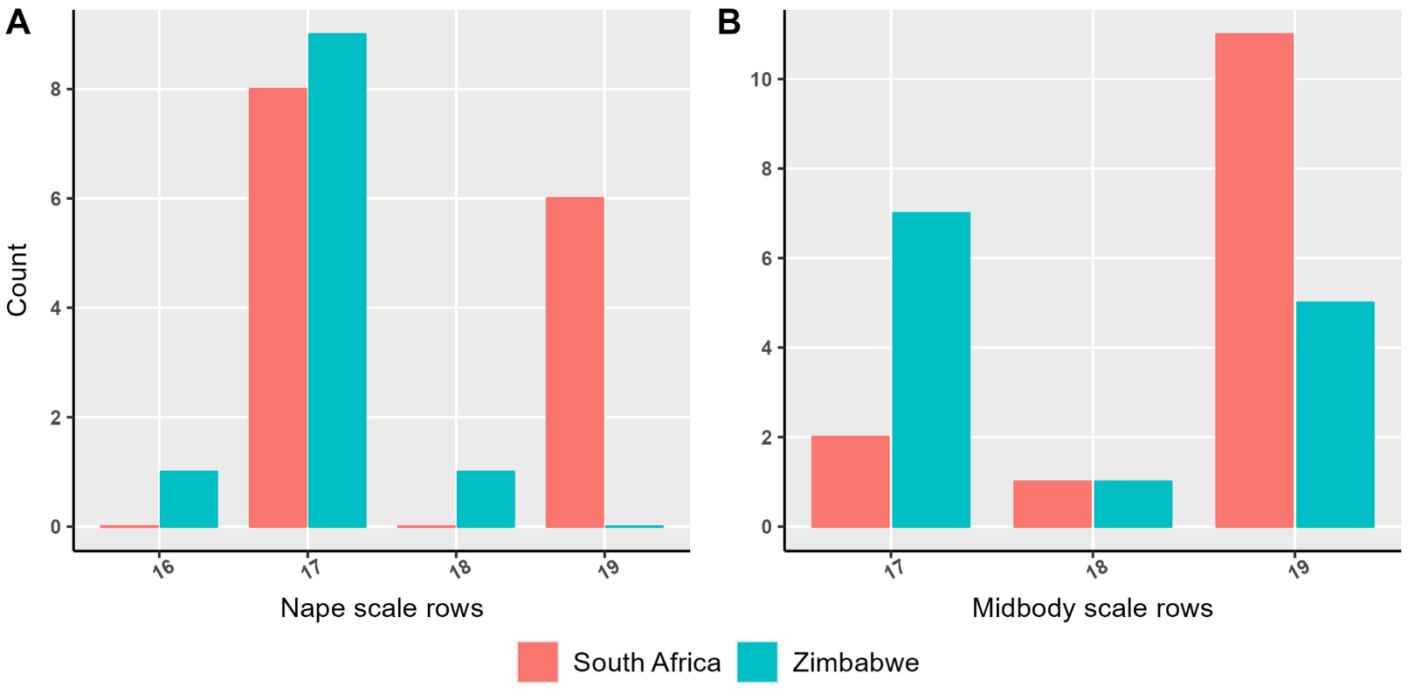

Figure 4.3: Bar plots showing the number of dorsal scale rows snakes possessed at the nape (A) and at midbody (B).

Table 4.3: Comparison of standard scale counts between *Hemachatus* from South Africa and Zimbabwe. Shown are the mean and standard deviation, with range in brackets, and sample size. Sexually dimorphic characters are divided by sex.

Character	South Africa	Zimbabwe
Midbody scale rows	18.6 ± 0.72 (17 – 19), *N = 14*	17.8 ± 0.95 (17-19), *N = 13*
Nape scale rows	17.9 ± 0.99 (17-19), *N = 14*	17 ± 0.43 (16-18), *N = 11*
Subcaudal scales females	39 ± 2.6 (35 - 40), *N = 6*	33.8 ± 2.3 (30 - 37), *N = 6*
Subcaudal scales males	40.9 ± 3.1 (35 - 46), *N = 14*	36.3 ± 1.8 (34 - 38), *N = 5*
Ventral scales females	141 ± 6.5 (129 - 148), *N = 5*	128 ± 1.6 (126 - 130), *N = 5*
Ventral scales males	129 ± 6.9 (117 - 138), *N = 13*	122 ± 1.7 (119 - 124), *N = 5*

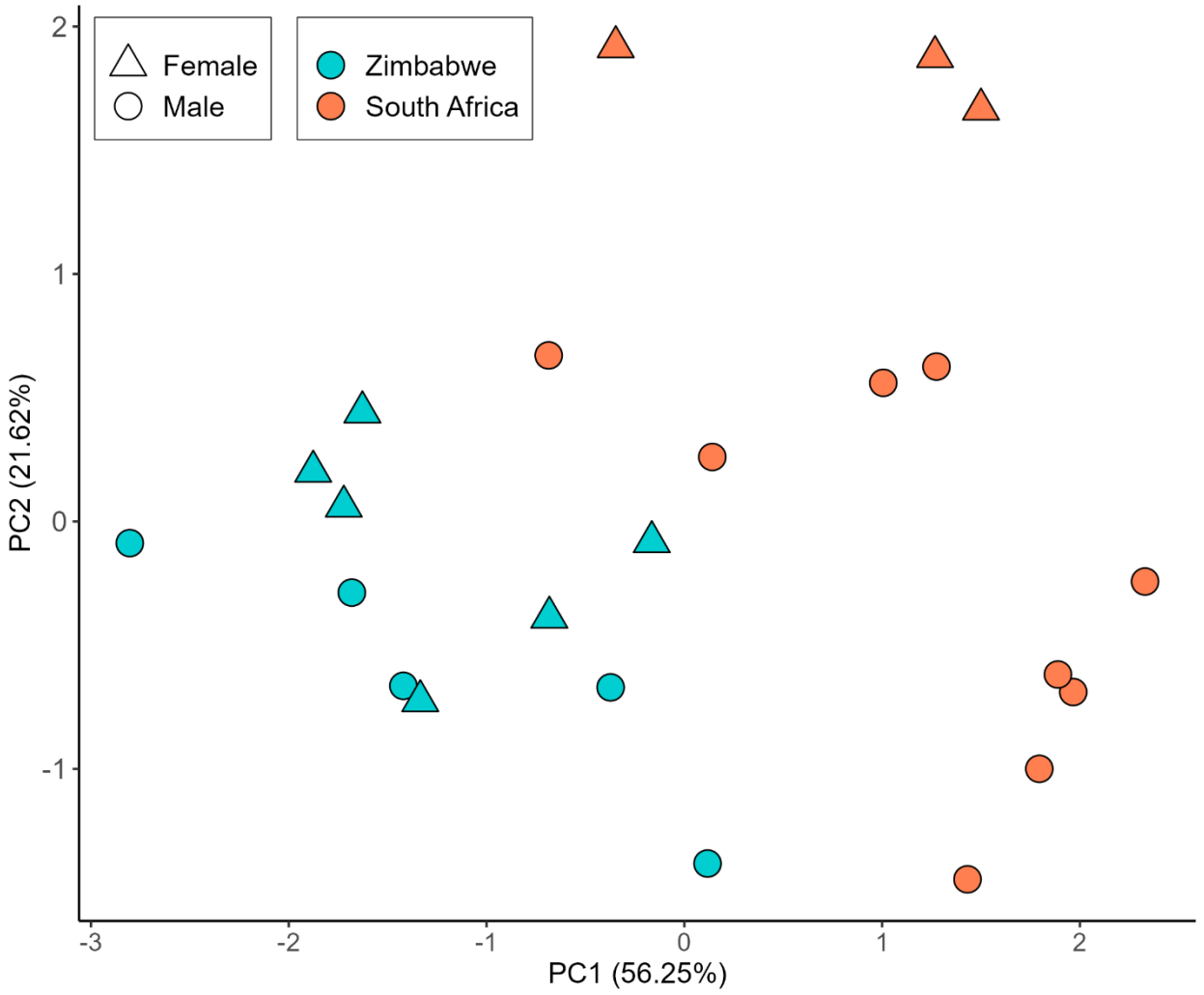

Figure 4.4: Ordination of South African and Zimbabwean specimens of *Hemachatus* along the first two principal components of the PCA of morphological data. Variables included are midbody scale rows, nape scale rows, number of ventral scales, and number of subcaudal scales. The first and second principal components represent 56.25% and 21.62% of the total variance in the data, respectively.

Table 4.4: Eigenvector coefficients for each scaled variable from the principal component analysis, eigenvalues, and the total variance explained by each principal component. The variables that contributed most to each principal component are in bold.

	PC1	PC2
Subcaudals	**0.576**	0.140
Ventrals	0.427	**0.774**
Nape rows	0.490	-0.545
Midbody rows	0.495	-0.291
Eigenvalue	2.245	0.865
Percentage of total variance	56.25	21.62

4.3.3 Species delimitation and systematics

The *Hemachatus* population in Zimbabwe is separated from the main range of *H. haemachatus* by over 700 km, and we used this separation to hypothesise that it may constitute a separate lineage (Figure 4.1). We tested for evidence of temporal lineage separation and found deep mtDNA sequence divergence between the Zimbabwe population and rinkhals from South Africa and Eswatini. Finally, phenotypic differentiation was confirmed using univariate analyses of standard morphological characters (Table 4.2). These lines of congruent evidence indicate that our candidate species, the *Hemachatus* population from Nyanga, Zimbabwe, represents an ancient lineage distinct from *H. haemachatus* in Lesotho, South Africa, and Eswatini (Figures 4.1 and 4.2), and therefore a separate species. As there are no published, available names for the Zimbabwean rinkhals, we here describe it as a new species.

***Hemachatus nyangensis* sp. nov.** Reissig, Major, Renk, Barlow, Paijmans, Morris, Hofreiter, Broadley, and Wüster.

Suggested common name: Nyanga rinkhals.

Zoobank ID: urn:lsid:zoobank.org:act:7BFD5FC9-556F-4C59-9246-83585E32E56D

4.3.4 Holotype

NMZB - UM 1307, a male specimen (Figures 4.5 and 4.6) collected from 2 kilometres northwest of Pungwe View, Nyanga National Park, Nyanga District, Manicaland Province, Republic of Zimbabwe, 18.42 °S, 32.78 °E, elevation 1122 m, by D. G. Broadley on 23/11/1961. This individual was killed by a car strike.

Dimensions: A male specimen, snout-vent length 635 mm, tail length 133 mm, total length 768 mm.

Body scalation: 18 scale rows around hood, 19 around midbody, 13 one head length ahead of the vent, all strongly keeled and oblique. Dorsal scales strongly keeled, oblique. Vertebral row not enlarged. 123 ventrals, 38 subcaudals, all divided except for first 4 which are single, anal single.

Head scalation: 7/7 supralabials, 3rd & 4th contact orbit, 5th largest; 8/8 infralabials, first 4 contact anterior chin shields; 1/1 preocular; 3/3 postoculars; 2/2 anterior temporals; 2/3 posterior temporals; rostral almost as deep as broad, clearly visible from above.

Pattern: Head black, body orange-brown with forty-three narrow black cross-bands, equal in width to the light interspaces, extending from just behind the neck till just behind the cloaca; chin dark brown, throat black with two white cross-bands, rest of belly and tail grey.

4.3.5 Paratype

NMZB 9503, a female specimen (Figure 4.6) collected on the grounds of the Nyanga Trout Hatchery, Nyanga National Park, Nyanga District, Manicaland Province, Republic of Zimbabwe, by G. Puttent in 1982. Cause of death is unknown but may have been killed by a person or struck by a vehicle.

Dimensions: A female specimen, snout-vent length 625mm, tail length 121mm, total length 746mm.

Body scalation: 17 scale rows around hood, 17 around midbody, 13 one head length ahead of the vent, all strongly keeled and oblique. Dorsal scales strongly keeled, oblique. Vertebral row not enlarged. 130 ventrals, 34 subcaudals, all divided, anal single.

Head scalation: 7/7 supralabials, 3rd & 4th contact orbit, 5th largest; 8/8 infralabials, first 4 contact anterior chin shields; 1/1 preocular; 3/3 postoculars; 2/2 anterior temporals; 3/3 posterior temporals; rostral almost as deep as broad, clearly visible from above.

Pattern: Head black, body yellow-brown with narrow black cross-bands, less distinct than in NMZB - UM 1307, and approximately equal in width to the light interspaces; chin dark brown, throat black with white cross-bands, rest of belly and tail grey.

Note: this is the specimen from which the DNA sequences used in this study were obtained.

4.3.6 Etymology

The specific epithet *nyangensis* means “from Nyanga” in Latin and is chosen to reflect the distribution of the species in the Nyanga district of Zimbabwe, the only area in which it has been documented.

4.3.7 Diagnosis

Distinguishable from its relative *Hemachatus haemachatus*, for which we propose the common name “Southern Rinkhals” and which occurs in South Africa, Lesotho, and Eswatini, by its isolated distribution in eastern Zimbabwe. Morphologically, *Haemachatus nyangensis* sp. nov. generally has overall lower body scale counts than its southern relative: it usually has fewer nape scale rows (16–18 instead of 17–19), midbody scale rows (commonly 17–19 vs usually 19) (Figure 4.3), fewer subcaudal scales in both females (30–37 vs 35–40 in *H. haemachatus*) and males (34–38 vs 35–46) and generally fewer ventral scales in both females (126–130 vs 129-148) and males (119-124 vs 117-138) (Table 4.3). The new species is genetically diagnosable through differences in the *12S* and *16S* mitochondrial sequence. The description of this species means that the genus *Hemachatus* is no longer monotypic.

4.3.8 Variation

Dorsal scale rows on neck 16-18, at midbody 17-19, before vent 11-13; ventrals 119-130, subcaudals 30-38, all divided (up to the first six rows of subcaudals can be singular). Males have lower ventral (119-124) and higher subcaudal (34-38) counts, whilst females have higher ventral (126-130) and lower subcaudal (30-37) counts. Supralabials 7 (rarely 6), the third and fourth (rarely third only) in contact with the orbit; infralabials 8 (rarely 7), the first 4 (rarely 3) in contact with the anterior chin shields; preocular 1; postoculars 3; temporals 2+2 or 2+3. The head is black, body yellow-brown or orange-brown, sometimes even reddish, with narrow black cross-bands, equal in width to the light interspaces. The black cross-bands can give the animal a clean appearance or sometimes even make it appear mottled. Some uniformly grey specimens have been recorded; chin dark brown, throat black, usually with two distinct white cross-bands, rest of belly and tail grey. The largest recorded individual was 865+150 = 1015 mm total length, from Nyanga National Park, Zimbabwe (NMZB – UM 16098). This new taxon has not been recorded spitting venom, despite having fangs modified for this behaviour (Broadley, 1961). This may be a result of the very small number of recorded interactions with humans.

4.3.9 Distribution and habitat

The distribution of *Hemachatus nyangensis* appears to be restricted to the immediate vicinity of the Nyanga National Park and Nyanga District in the Eastern Highlands of Zimbabwe. It has been recorded from montane grasslands with areas of miombo woodland (Figure 4.5). The Eastern Highlands run along the eastern edge of Zimbabwe and into Mozambique, forming part of southern Africa's Great Escarpment. Containing numerous endemic animal and plant species (Clark et al., 2017), the Highlands are included within the Eastern Afromontane Biodiversity Hotspot, an area with high potential for undiscovered biodiversity (Mafuwe & Moyo, 2020). It is possible that this species also occurs in neighbouring Mozambique, as both the habitat and climatic conditions are similar.

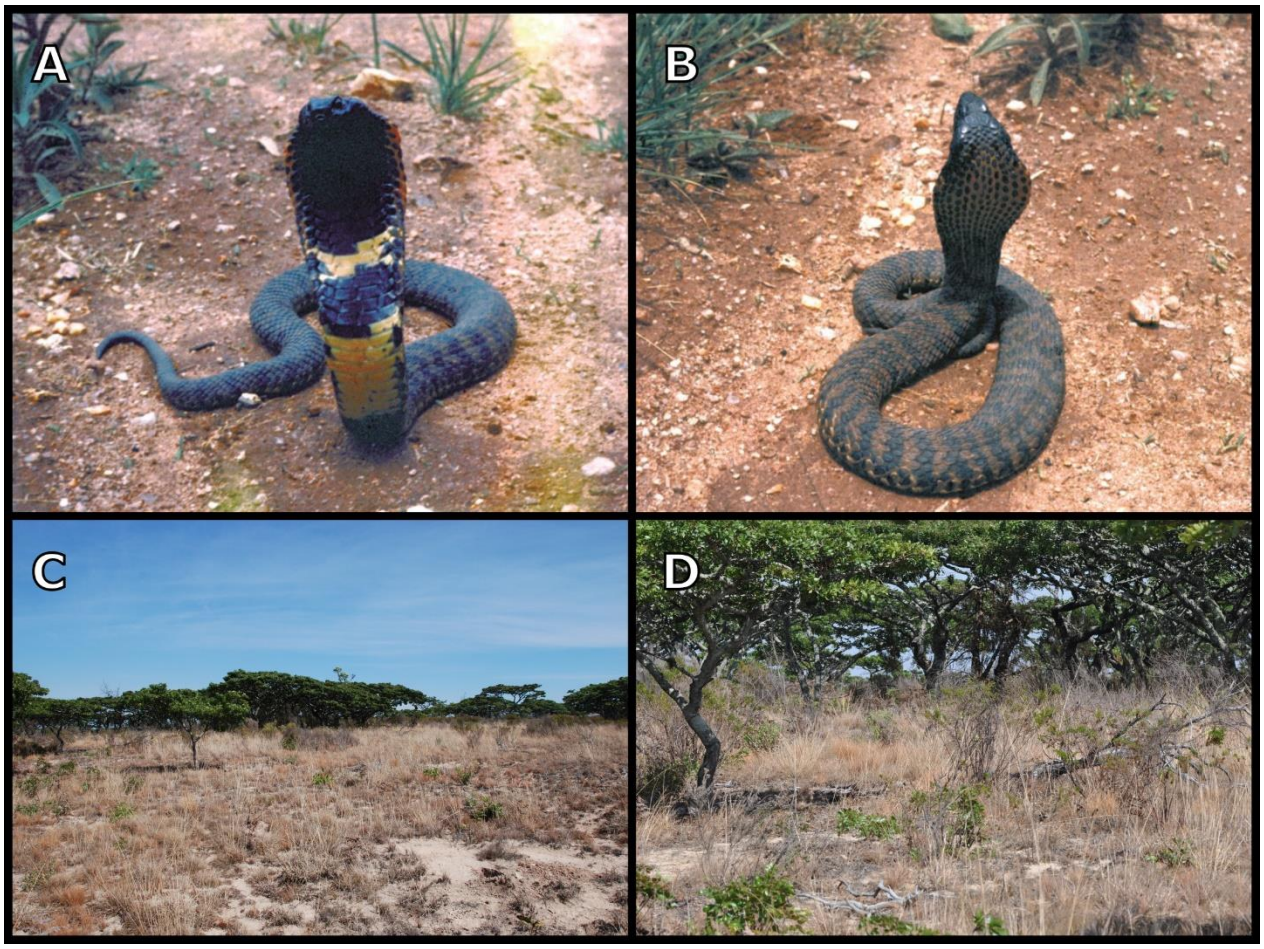

Figure 4.5: (A and B) *Hemachatus nyangensis* sp. nov. specimen in life, displaying defensive hooding posture. (C and D) Miombo woodland and grassland habitat of *H. nyangensis* sp. nov.

Figure 4.6: (A) Male holotype specimen of *Hemachatus nyangensis* sp. nov. from Nyanga National Park, following preservation (NMZB - UM 1307). (B) Female paratype specimen of *Hemachatus nyangensis* sp. nov. from Nyanga National Park, following preservation (NMZB 9503).

4.4 Discussion

4.4.1 Molecular dating and biogeography

Our dated phylogeny of *Hemachatus* and related elapids corresponds largely (except for the interrelationships of the subgenera of *Naja*) to the tree of Kazandjian et al. (Kazandjian et al., 2021). Despite very obvious regional differences in colour pattern (Branch, 1992; Alexander & Marais, 2007), the rinkhals populations across southern Africa displayed little mitochondrial divergence, with diversification within *H. haemachatus* beginning around 0.69 Mya. Conversely, we estimate that the *H. haemachatus* clade from southern Africa and its sister lineage, *H. nyangensis* from Zimbabwe, diverged approximately seven to 14 million years ago. *Hemachatus* are temperate adapted (Alexander, Mitchell & Hanrahan, 1999). Following the mid-Miocene Climatic Optimum 17-15 Mya, global temperatures cooled significantly (Zachos et al., 2001). During this period of cooling the Antarctic ice-sheet was re-established by 10 Mya, and the Arctic ice-sheet formed for the first time by 3.2 Mya (Zachos et al., 2001). On this cooling Earth, southern Africa was becoming more arid, due in part to cold water flowing northward along the western shore of southern Africa after the opening of the Drake Passage between South America and Antarctica (Rommerskirchen et al., 2011). The increasing aridity of lowland areas is likely to have caused significant range changes and fragmentation, particularly in temperate adapted species (Tolley, Chase & Forest, 2008; Channing et al., 2022). Indeed, a dated phylogeny for *Strongylopus* frogs suggests a split

between *S. rhodesianus* in the Eastern Highlands and the ancestor of *S. fasciatus* and *S. merumontanus* approximately 16 Mya (Channing et al., 2022). Similar vicariance can be observed in *Bradypodion* chameleons in the mid to late Miocene (Tolley, Chase & Forest, 2008), and the tortoise species *Psammobates tentorius* (Zhao et al., 2020)). The same fragmentation likely affected the ancestors of *H. haemachatus* and *H. nyangensis*. Areas of suitable habitat across southern Africa shrank as it became drier, with lowland areas becoming subtropical, splitting *Hemachatus* between the temperate Eastern Highlands and the southern temperate biogeographic zone. Indeed, the Nyanga Massif where *H. nyangensis* is found is a close-knit bundle of mountains containing Zimbabwe's highest point, Mount Nyangani, and most of the Massif is over 2000 metres above sea level. As a result of this high elevation, the Nyanga Massif has a cooler and wetter climate than the surrounding lowlands. At least two other temperate-adapted snake species, the viperid *Bitis atropos* and the lamprophiid *Amplorhinus multimaculatus*, display a similarly fragmented distribution as *Hemachatus*, with isolated populations in the Eastern Highlands; both warrant further systematic and phylogeographic attention.

4.4.2 Implications for the evolution of spitting in elapid snakes

Based on molecular dating of the initial divergence of spitting cobras in Africa (subgenus *Afronaja*) and Asia (spitting clade of the subgenus *Naja*), Kazandjian et al. (2021) proposed that this defensive adaptation may have arisen as a result of the evolution of early hominins in Africa and their later arrival in Asia. *Hemachatus* was uninformative with respect to this hypothesis, as the origin of spitting was unconstrained along the long branch leading to a monotypic *Hemachatus*, offering little insight into when spitting emerged in this group. The discovery of a highly divergent new species in the genus allows the minimum age of the timing of the evolution of spitting in this lineage to be re-evaluated. Our point estimate of 10.14 Mya for the divergence of Zimbabwean and southern African *Hemachatus* significantly predates the estimated divergence between the lineages leading to *Pan* and *Homo* at 7.65 Mya (Pozzi et al., 2014), but our 95% HPD of 6.25-14.39 Mya encompasses the point estimate and the entire 95% credibility interval of that study (6.73-8.76 Mya).The reliance on short fragments of *12S* and *16S* rDNA sequence necessitated by poor preservation of the Zimbabwean rinkhals sample, and the resulting broad maximum credibility intervals preclude robust inferences on the timing of the focal node and any association with potential selective drivers for spitting. However, the apparent great age of the *H. haemachatus* - *H. nyangensis* divergence suggests that this taxon could be of considerable interest in the investigation

of the potential drivers and correlates of the evolution of spitting. If *H. nyangensis* is extant, fresh material would provide the data required to more rigorously test the hypothesis of an association between hominin origins and the evolution of spitting in this third spitting elapid lineage.

4.4.3 Museum DNA, species descriptions, and the future of the Nyanga rinkhals

We have provided proof of concept that, with appropriate museum genomic methods, even extremely low-quality museum samples can yield enough DNA sequence to infer the status of an undescribed species. Here, it has allowed us to identify and describe a cryptic and potentially extinct species of snake. The Eastern Highlands are under pressure from habitat modification by agriculture, illegal logging, and small-scale gold mining activities, and as a result of these factors the area has changed dramatically since the 1980s (Mafuwe & Moyo, 2020). Invasive plant species are also modifying the montane grassland of the Nyanga Massif (Clark et al., 2017). While *H. nyangensis* has not been seen since 1988, Broadley and Blaylock (Broadley & Blaylock, 2013) suggest it should be searched for in remaining unaltered habitat in the eastern parts of Nyanga National Park. In recent years there have been a few notable cases of species which were thought to be extinct being rediscovered (Scheffers et al., 2011; Glaw et al., 2020), including Jackson's climbing salamander (*Bolitoglossa jacksoni*), Wallace's giant bee (*Megachile pluto*), and Voeltzkow's chameleon (*Furcifer voeltzkowi*). The description of *H. nyangensis* sp. nov. draws attention to the importance of Zimbabwe's Eastern Highlands as a region of high biodiversity and endemism. We remain hopeful that the species will eventually be rediscovered in the less anthropised parts of the Nyanga Highlands and strongly encourage renewed fieldwork to confirm its existence. Much of the area is Nyanga National Park, incorporating an area of 472 km^2 and encompassing half of the Nyanga Massif, hopefully affording the species some protection if it is extant. Understanding the ecological requirements of this species (climate, diet, habitat structure) is essential for an assessment of its conservation status and the formulation of a recovery plan. Additional genetic sampling and sequencing will help resolve the status and origin of this mysterious snake, and contribute to our understanding of the biogeographic significance of the Eastern Highlands of Zimbabwe. It will also help to clarify the ecological context of the origin of venom spitting, and the role our ancestors might have played in driving this defensive innovation.

4.5 Acknowledgements

We thank the late Tony Phelps for his contribution to this research. For permission to sample specimens of *Hemachatus* in their care, we thank Thea Litschka Koen and Clifton David Koen, Mark Marshall, Arno Naude, Mike Perry, and Moshe Kahn. Krystal Tolley kindly provided access to laboratory facilities in Cape Town, as well as insightful reviews of two earlier drafts of this manuscript, which greatly improved the final version.

Chapter 5: General discussion

We investigated the status of the isolated rinkhals population from Nyanga, Zimbabwe using both phylogenetic and morphological analyses in an integrated taxonomic framework. While we anticipated the population would show some divergence from snakes from southern Africa, we were surprised to discover that the population has been separated for approximately 10 million years. The genetic and morphological differences led to us describing it as the Nyanga rinkhals (*Hemachatus nyangensis*), separate from the southern (formerly common) rinkhals (*Hemachatus haemachatus*). This population of snakes, isolated from their congeners in southern Africa, have adapted to their local environment, and we have determined them to be a distinct lineage worthy of species status. The Nyanga rinkhals therefore demonstrates the most extreme form an isolated population can take – speciation. An assessment by the International Union for the Conservation of Nature is currently underway to determine whether this species warrants inclusion on the Red List, and it is likely to be listed as Critically Endangered (Pietersen, Wüster & Alexander, in prep). Further surveys are required to determine if they remain extant.

The circumstances leading to the isolation of the Nyanga rinkhals are climatological and natural rather than human mediated. Their isolation was a far more ancient event compared to the nascent, human-mediated introductions of the Aesculapian snake in the UK. Despite this, the two species face broadly similar challenges from limited habitat availability, as well as genetic isolation. We found that introduced and relict Aesculapian snake populations showed genetic symptoms of recent bottlenecks. Additionally, our radio-tracking study revealed a suite of behavioural adaptations to a colder climate by Aesculapian snakes in North Wales, suggesting a reliance on human features of the habitat in Colwyn Bay.

5.1 Genetic consequences of isolation

While the factors leading to the genetic isolation are different, severe genetic bottlenecks occur in both naturally isolated populations and populations introduced in small numbers to new areas by humans. Aesculapian snakes were once native to Britain, but subsequently went extinct (Musilová et al., 2010). There remains uncertainty around the geographic origins of the Welsh population, and the number of founding individuals that began both the UK populations, but their reappearance provides an opportunity to study the dynamics of two nascent introductions. Isolation and the resulting restriction in gene flow is a known cause of deterioration in wild populations (Naimo et

al., 2021). This can result in reduced adaptive potential, or even more dramatic cases involving the preponderance of morphological abnormalities (Miller et al., 2011). The Iberian lynx (*Lynx pardinus*) has the lowest genome-wide genetic diversity reported for any species, and represents the extreme of inbreeding and genetic erosion (Abascal et al., 2016). This has resulted in a high frequency of genetic diseases, conditions such as epilepsy, and a high proportion of abnormal sperm. This has been somewhat alleviated by artificially admixing the two remaining populations (Abascal et al., 2016). We discovered unexpectedly high levels of heterozygosity in the Welsh population of Aesculapian snakes. However, an individual from a native population close to Rome, and an individual from a relict population in Germany, showed high levels of inbreeding consistent with their apparent isolation. For these populations, genetic rescue may be a relevant measure to ensure their future viability if natural connectivity remains impossible. One concern of mixing disparate populations is that they may practice assortative mating, where newly mixed populations do not willingly reproduce with each other. In the North Island brown kiwi, (*Apteryx mantelli*), a bird from New Zealand, the opposite was found to be true – birds were more likely to mate with birds from a different source population than would be expected by random chance, suggesting a possible mechanism to reduce inbreeding (Undin et al., 2021). Encouragingly, a meta-analysis suggested that the benefits of genetic rescue far outweigh the costs in the majority of cases, especially where animals had been screened as having a low risk of outbreeding depression (Frankham, 2015). While this can be a source of optimism for genetic rescue efforts, there remains caution in the conservation community because of the risk of genetic homogenisation, outbreeding depression, or loss of local adaptation (Kardos et al., 2018; Bell et al., 2019).

While the population in London showed signs of severe inbreeding and a strong genetic bottleneck, the population in Wales had surprisingly high levels of genome-wide diversity. While their origin remains unclear, if the Aesculapian snakes in Colwyn Bay are of mixed ancestry, it could provide greater adaptive potential (Hunter et al., 2018). In the case of the Asiatic wild ass (*Equus hemionus*) reintroduced to Israel, a breeding core of individuals was created from two different subspecies (Zecherle et al., 2021). Despite the fears of conservationists, the population has grown quickly and displays no signs of outbreeding depression, and this mixing has been credited with increasing their genetic diversity following the conservation translocation (Zecherle et al., 2021). If the Aesculapian snakes in Colwyn Bay are similarly admixed from distant populations, this may be conferring an adaptive advantage, with higher genetic diversity known to be beneficial for re-introduced tortoises (Scott et al., 2020). Conversely, outbreeding can have negative consequences for populations which

have experienced long-term genetic homogenisation because of highly inbred mating systems. Social spiders show reduced hatching success when outcrossed, despite a lack of difference in offspring performance (Berger-Tal et al., 2014). Despite the limitations of a lack of genetic variation, many invasive species adapt rapidly to their new environments. One little explored explanation for this may be transposable elements, known as 'jumping genes', within their genomes. These are mobile parasitic elements that propagate themselves in the genome, with a wide variety of effects including new mutations and reversed mutations (Fueyo et al., 2022). They are known to have allowed adaptation in mammals over evolutionary timescales (Fueyo et al., 2022), and could be responsible for rapid adaptation in introduced species (Stapley, Santure & Dennis, 2015), potentially conveying benefits to isolated populations.

5.2 Roads and isolation

Roads are known to be a major causative factor in the genetic isolation of many species (Epps et al., 2005), including reptiles (Bauder et al., 2021). Even railroad tracks are sufficient to form barriers for less mobile species such as salamanders (Bartoszek & Greenwald, 2009). Wildlife overpasses have been used to provide connectivity between patches of habitat, but there has been limited evidence showing support to the notion that they can increase admixture between populations (Corlatti, Hackländer & Frey-Roos, 2009). However, recent investigations into wild boar (*Sus scrofa*) suggest an increase in genetic mixing resulting from the construction of an overpass, when compared to populations which are still separated by a highway (Kupferschmid et al., 2022). For snakes, underpasses may be more suitable, and king cobras (*Ophiophagus hannah*) have been shown to make repeated use of the same underpasses (Jones et al., 2022). In the UK, while thought is given to wildlife when constructing infrastructure, it is seldom evidence-based, and many projects do not make any allowances for wildlife. Such considerations are likely to be crucial to the long-term genetic health of animal populations, even for highly mobile species. We witnessed numerous road deaths for Aesculapian snakes. Snakes in our radio-tracked sample were unwilling to venture over roads, and those that did suffered fatal consequences. Therefore, means of by-passing roads must be considered both for the short-term survival of individuals, and for the long-term maintenance of genetically healthy populations.

5.3 Shifting reptile populations

All animals have thermal tolerances which limit the areas they can survive in, after accounting for behavioural thermoregulation (Parlin, Schaeffer & Jezkova, 2020). Over time, the ranges of animals shift in response to climatological factors, as the changing climate permits different areas of the planet to be suitable for their needs (Bellard et al., 2012). Historical climate change has been responsible for the isolation and speciation of many reptile species. The Nyanga rinkhals became isolated from its congener the southern rinkhals (*Hemachatus haemachatus*) due to aridification of southern African over millions of years, leading to high-altitude isolation in the relatively cool the Eastern Highlands. Conversely, Aesculapian snakes at their northern range limit represent populations made relict by a retreat at the northern range limit as temperatures cooled. Northern populations in Germany, Czechia, and Poland are isolated to small pockets of suitable habitat that are thought to offer localised suitable habitat and warmer thermal regimes (Kovar et al., 2016; Kurek et al., 2019).

Reptiles have experienced shifts in their ranges throughout the quaternary, generally expanding during warmer periods and contracting during cooler ones (Martínez-Monzón et al., 2021). In modern times, one of the most profound effects of human-mediated climate warming is the shift poleward and to higher altitude occurring in the ranges of many species (Lenoir & Svenning, 2015). This is resulting in range expansions at their cool extremes, and contractions in hotter areas closer to the equator (Biber, Voskamp & Hof, 2023). Accordingly, the changing climate may increase the suitable habitat available to many of Europe's reptiles. However, reptiles are generally poor dispersers (Sahlean et al., 2014), leaving them unable to exploit the newly available habitats. This is likely to be exacerbated in Europe where habitats are already highly fragmented (Araújo, Thuiller & Pearson, 2006). For many species, climate refugia have been considered in areas which are less vulnerable to the most extreme effects, although these most frequently involve providing corridors to enable animals to travel themselves (Fischman et al., 2014). There is a growing body of literature regarding assisted migration for the conservation of native species under rapid climate change (Butt et al., 2021), especially for narrow endemics which have little dispersal possibility (Thomas, 2011).

Often, species are reintroduced with the intention of conserving them, or benefiting the local ecological community (Hunter-Ayad et al., 2021). These introductions can be performed legally as part of sanctioned re-introduction projects (Rehm et al., 2018; Zhang et al., 2019), or as part of so-called 'clandestine' introductions, where individuals release species to foster new populations that

they perceive to be of conservation value (Vaccaro & Beltran, 2009; Drouilly & O'Riain, 2021). Of course, such operations remain shrouded in secrecy, but there are vocal proponents. In the age of social media, it is conceivable that they may become more common as human beings can become quickly and determinedly allied to causes following the uptake of persuasive media. In the UK, there are already countless small populations of introduced mainland European reptiles, probably introduced by enthusiasts, largely existing in isolated locations close to urban areas (Michaelides et al., 2015). Similarly, the pine marten (*Martes martes*) has been expanding since lows before the 1980s, and while there has been successful conservation work for pine martens in Scotland and Wales, populations in southern England apparently arrived without legitimate help (Sainsbury et al., 2019). Likewise, some polecat releases in southern England have been attributed to clandestine actors due to their fur colour (Birks, 2008). Eurasian otters (*Lutra lutra*) in the UK show a genetic signature of Asian origin. Ex-situ efforts attempt to breed and release animals of local provenance from Europe, so Asian genomic influence is unexpected - this could result from an accidental mating with an Asian specimen during ex-situ conservation work, or a clandestine release of Eurasian otters of Asian origin (du Plessis et al., 2023). While clandestine release activities may have local benefits, when undertaken without proper planning they could result in severe repercussions for poorly understood ecosystems.

Sanctioned conservation translocations are usually conservative, where candidate release sites are chosen to reflect conditions where the species is currently found, without considering past declines or range changes (Hunter-Ayad et al., 2021). The alternative to this is using an extrapolative strategy, where sites for introductions are chosen using data sources from outside the current populations. This information can be gathered from trophically similar species, using information from captive animals, or by comparing the range of a closely related sister-species (Hunter-Ayad et al., 2021). Of course, the best possible information comes from studying the species in question and thoroughly understanding how it uses habitat, especially during crucial behaviours such as oviposition and hibernation. The Aesculapian snake is considered Critically Endangered in Germany (Rohde-Fingerle, Matzke-Hajek & Broghammer, 2020) and protected by the EU habitats directive and the Bern convention. Despite this, the Aesculapian snake is considered least concern by the IUCN due to its broad distribution (Agasyan et al., 2016). While it is perhaps not an obvious target for the planning of climate refugia, this could change if southern and low-lying parts of the range become inhospitable due to climate change. Indeed, a new classification for non-native and introduced species puts less emphasis on what was naturally present, and focuses on the nuance of

an animal's effect within the ecosystem (Lemoine & Svenning, 2022), although crucially the effects of Aesculapian snakes on other UK wildlife are not clear.

5.4 Behaviour of introduced species

We have shown that the Aesculapian snake in the UK makes heavy use of buildings and compost heaps in a semi-urban area. The Aesculapian snake has successfully established itself in two areas, and like so many other novel species, one is extremely urban – Central London incorporating London Zoo. It is clearly able to adapt to a lifestyle incorporating time spent beneath and within structures created and often occupied by human beings. This may be key to their success at higher latitudes. Artificial structures can serve as stepping stones for species' which are expanding their range into areas which were previously unsuitable (Cannizzo and Griffen, 2019). The abundance of warm buildings, the city warming effect, and the fact that there are heated exhibits for tropical species in both locations where they are found could be contributing to the success of Aesculapian snakes. For egg-laying reptiles, oviposition and hibernation are two of the foremost challenges in any environment. Our discovery that Aesculapian snakes use gardens and buildings for these activities implies they may be crucial to their survival in the UK. Further study of populations across the temperature cline that the species naturally inhabits would better contextualise the nature of their behaviour in the UK.

5.5 Control of Aesculapian snakes

The UK's House of Commons Environmental Audit Committee consider the Aesculapian snake a potential priority for rapid eradication, estimating a cost of £75,000 to perform the eradication (House of Commons Environmental Audit, 2019). Despite this, no action has been performed on either population. If deemed necessary in the future, attempts to control Aesculapian snakes should focus on manual capture during periods of greatest activity. The results of our motion variance analysis suggest that for adult males, high activity levels during the mating season from mid-May until the end of June are likely to expose the snakes to capture on the surface. Similarly, gravid females are likely to be more active during July and August when they must seek out suitable oviposition sites. Searches targeting females and eggs in likely egg-laying sites such as compost heaps, vegetation piles, and underground crevices in the area surrounding the Welsh Mountain Zoo during this period would also be a sensible approach to management. If adult females can be found, their removal would likely greatly impact Aesculapian snake numbers. Although the use of

acetaminophen-laced bait remains untested in Aesculapian snakes, it may also be effective because Aesculapian snakes readily eat rodents, (Goetz, Yackel Adams & Siers, 2020). However, to ensure native species are not affected, traps containing baits must be designed to exclude other wildlife.

5.6 Limitations

Snakes are cryptic and secretive predators and little is known about their behaviour, even for the largest and most charismatic species (Smith et al., 2021). Radio telemetry is an extremely labour-intensive means of gathering information about the behaviour of animals (Boback et al., 2020). The lack of appendages on snakes precludes traditional attachment methods, and requires internal attachment in most cases (Reinert & Cundall, 1982). We experimented using external attachment of radio transmitters on Aesculapian snakes, but the snakes always removed them within three days (n = 3). Modern approaches increasingly utilise the power of GPS technology to determine the movements and behaviours of animals (Brown et al., 2012; Bischof et al., 2019; Forrest, Recio & Seddon, 2022). Until recently, GPS tracking devices were only suitable for relatively large snake species, owing to the size of the attachment package (Smith et al., 2018). Because of this, GPS technology has received very little use in snakes, with a few notable exceptions (Wolfe, Fleming & Bateman, 2018; Whitney et al., 2021; Gerke, Hinton & Beasley, 2021). Of these few examples, only Burmese pythons have had GPS tags internally attached, and this still requires a bulbous antenna positioned on the exterior of the snake (Smith et al., 2018). Additionally, fix rates are greatly reduced if snakes spend long periods underwater, underground, or in dense vegetation (Smith et al., 2018) – all behaviours commonly exhibited by snakes. Because of this, the use of GPS and VHF in conjunction will likely be beneficial for when snakes are spending long periods hidden. Fortunately, GPS tracking devices are becoming rapidly miniaturised, and tags weighing as little as one gramme are now commercially available for tracking a suite of animals (Wild et al., 2022). This will permit attachment to even small species of snake, provided external attachment is appropriate. When the animal is in sight of the sky, the automated data collection of GPS devices allows for more many more fixes on the location of the animal. This is extremely beneficial for applications such as step-selection functions which examine movement decisions (Thurfjell, Ciuti & Boyce, 2014). Because of this, there is likely to be a revolution in the application of tracking approaches used on snakes when the bulky exterior GPS antennae can be further miniaturised or removed.

5.7 Summary and recommendations

With road mortality apparently so high in this population, and the use of underpasses by snakes previously documented (Kovar et al., 2014; Jones et al., 2022), it would be prudent to build underpasses into as many roads as possible. While long underpasses may deter reptiles due to inadequate thermal conditions (Remon et al., 2022), Aesculapian snakes use underpasses to traverse their native range (Kovar et al., 2014). As roads have been implicated in pronounced gene-flow reduction in grass snakes (*Natrix helvetica*), a UK native, increasing the permeability of the landscape should be advantageous for UK reptile species too (Remon et al., 2022).

We found that the Aesculapian snake is making heavy use of human features, and these structures likely provide multiple advantages. If the snakes are using the buildings to attain suitable body temperatures, this would greatly inform their likelihood of spreading. Human habitat features might be crucial to Aesculapian snake survival at high latitudes, or simply one of a suite of options open to an adaptive species. Investigating the thermal ecology of the species in their novel environments in the UK will determine what benefit the snakes are receiving and should underscore future work. Additionally, behavioural study of native populations at their northern and southern range limits would cast light on how behaviour varies in different climatic regimes, particularly in isolated and relict populations in Germany, Czechia, and Poland. This would be useful in determining whether conservation actions such as climate translocations would be beneficial, and the kinds of areas which represent suitable habitat.

Because of the difficulty in sourcing genetic material from isolated and rare species, we used an old museum specimen dating from the early 1980s to conduct our genetic investigation into the Nyanga rinkhals. Although data from further mitochondrial gene regions were available from other species for comparison, only two relatively short sequences from the *12S* and *16S* regions of the mitogenome were used in describing this species. While enough to delineate the species, this precluded a more precise timing of the split between *H. haemachatus* and *H. nyangensis*. Fresh genetic material from the isolated Nyanga rinkhals may shed light on the evolutionary history of the group, as well as the evolution of spitting in Elapid snakes.

In terms of the ancestry of the Aesculapian snake population in Colwyn Bay, further work should seek to determine their origins, and whether they have mixed ancestry. If they do, it may be conferring a selective advantage (Zecherle et al., 2021). The nature of this admixture would be

informative for the conservation of northern relict populations, who are similarly surviving at the northern extreme of the species' range limit.

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Appendices

Co-author contributions

Chapter 2: Benjamin Michael Marshall contributed to study design and modified figures. Lauren Jeffrey created the map figure. Writing and review contributed by Lauren Jeffrey and Prof. Wolfgang Wüster.

Chapter 3: Genetic library preparation and lab work performed by Dr Daniel Förster. Genome assembly script written by Dr Johanna Paijmans. Lab work assisted by Ben Owens. Writing and review contributed by Axel Barlow and Prof. Wolfgang Wüster.

Chapter 4: Ancient DNA extraction and processing performed by Prof. Michael Hofreiter, Dr Johanna Paijmans, Pia Renke, and Dr Axel Barlow. Ancient DNA methods prepared by Dr Axel Barlow. Final phylogenetic analysis performed by Prof. Wolfgang Wüster. Species description written by Jens Reissig. Modern DNA extractions conducted in collaboration with Ellie Morris. Writing and review contributed by Axel Barlow and Prof. Wolfgang Wüster.

Chapter 2: Supplementary material

Table S2.1: Capture records and time in captivity for radio-tracked Aesculapian snakes in this study, with additional movement metrics, and relocations. MDD = mean daily displacement.

ID	Capture date	Released	Total distance moved (m)	MDD (m)	Maximum daily distance (m)	Relocations
F050	13/06/2022	17/06/2022	216.20	7.74	56.78	13
F142	24/05/2021	03/06/2021	264.26	8.05	58.09	20
F158	22/06/2022	24/06/2022	1028.29	20.57	92.10	31
F159	11/06/2021	18/06/2021	3673.04	60.38	364.43	47
F177	07/07/2021	21/07/2021	782.51	8.68	44.67	47
F203	11/05/2022	13/05/2022	1282.96	19.70	72.06	60

F212	24/05/2022	27/05/2022	157.93	38.04	64.73	8
F219	25/06/2022	29/06/2022	555.29	46.00	244.47	7
M031	03/06/2022	07/06/2022	1135.56	34.20	129.11	45
M073	10/05/2021	02/06/2021	698.28	78.71	206.39	8
M074	14/07/2022	15/07/2022	179.99	5.97	64.96	6
M137	10/05/2021	02/06/2021	3894.85	30.95	382.88	76
M139	15/05/2021	03/06/2021	3903.90	41.10	559.30	64
M149	02/06/2021	04/06/2021	1706.27	46.37	336.90	28
M154	07/06/2022	17/06/2022	2427.55	32.70	107.16	75
M178	25/07/2021	28/07/2021	999.82	12.04	255.31	25
M180	29/07/2021	05/08/2021	1225.27	21.54	125.12	21
M202	08/05/2022	11/05/2022	7935.06	70.85	566.25	142
M209	20/05/2022	25/05/2022	7387.04	69.03	365.16	126
M217	11/06/2022	14/06/2022	1477.92	86.94	383.95	23
M218	21/06/2022	24/06/2022	4469.91	66.73	586.54	75

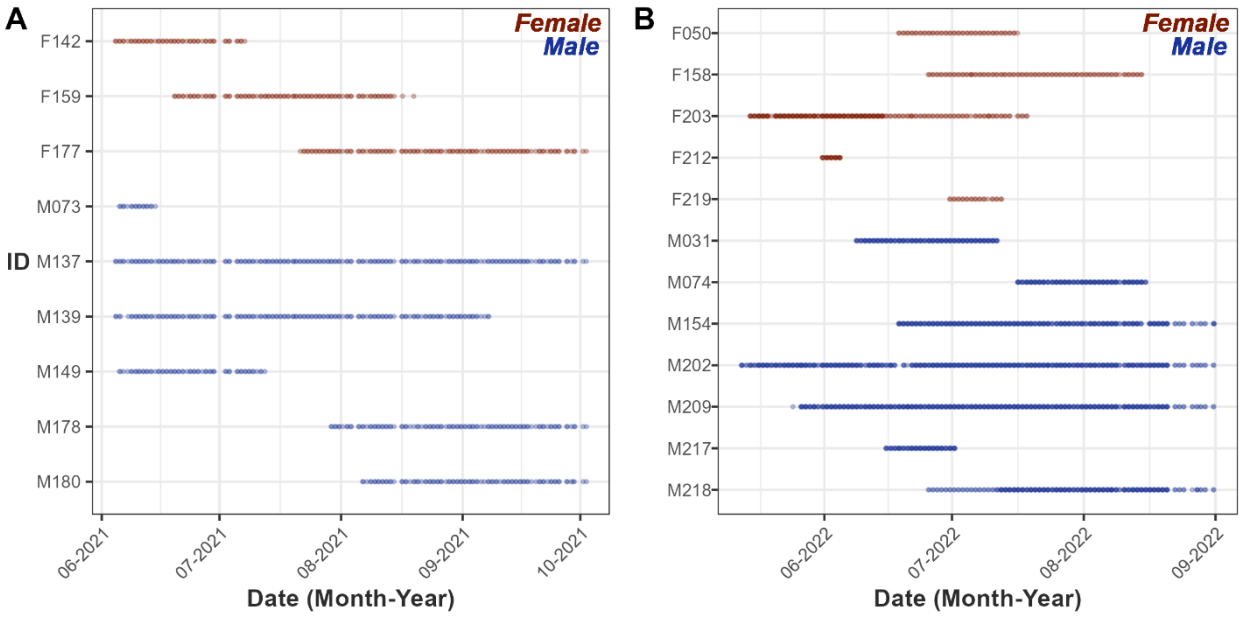

Figure S2.1: Tracking periods for 21 tracked snakes over (A) 2021 and (B) 2022. Each point represents a tracking occasion where the snake was located, with a higher density of points representing increased tracking frequency.

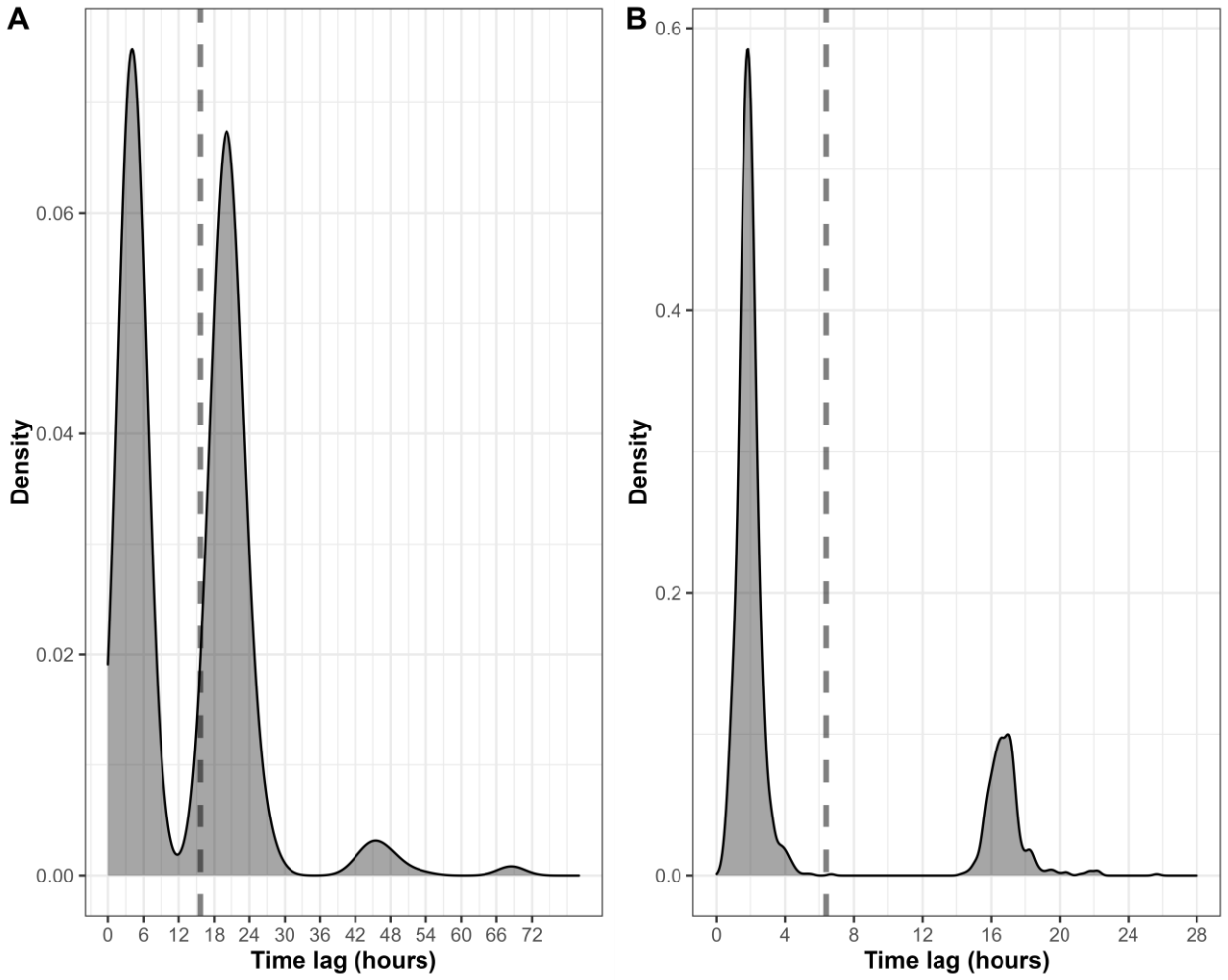

Figure S2.2: Time lag between tracks for snakes which were tracked A) twice daily and B) five times daily. Both show a first peak between the tracks during a day, and a second which represents the gap overnight. The checked line marks the mean time lag between tracks.

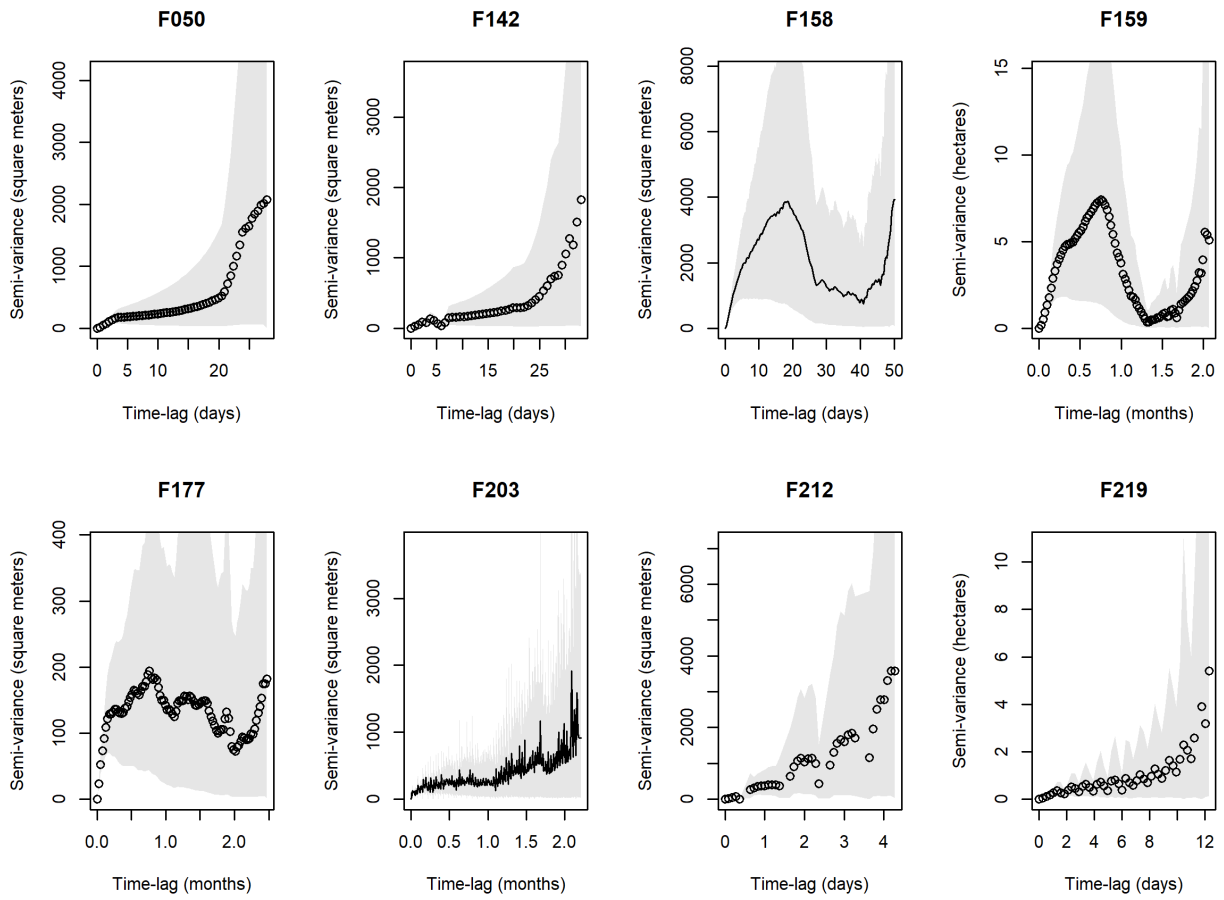

Figure S2.3: Variogram plots visualising the semi-variance function for tracked female snakes. F158, F159 and F177 can be said to have reached range residency as the plots reach an asymptote and stop rising.

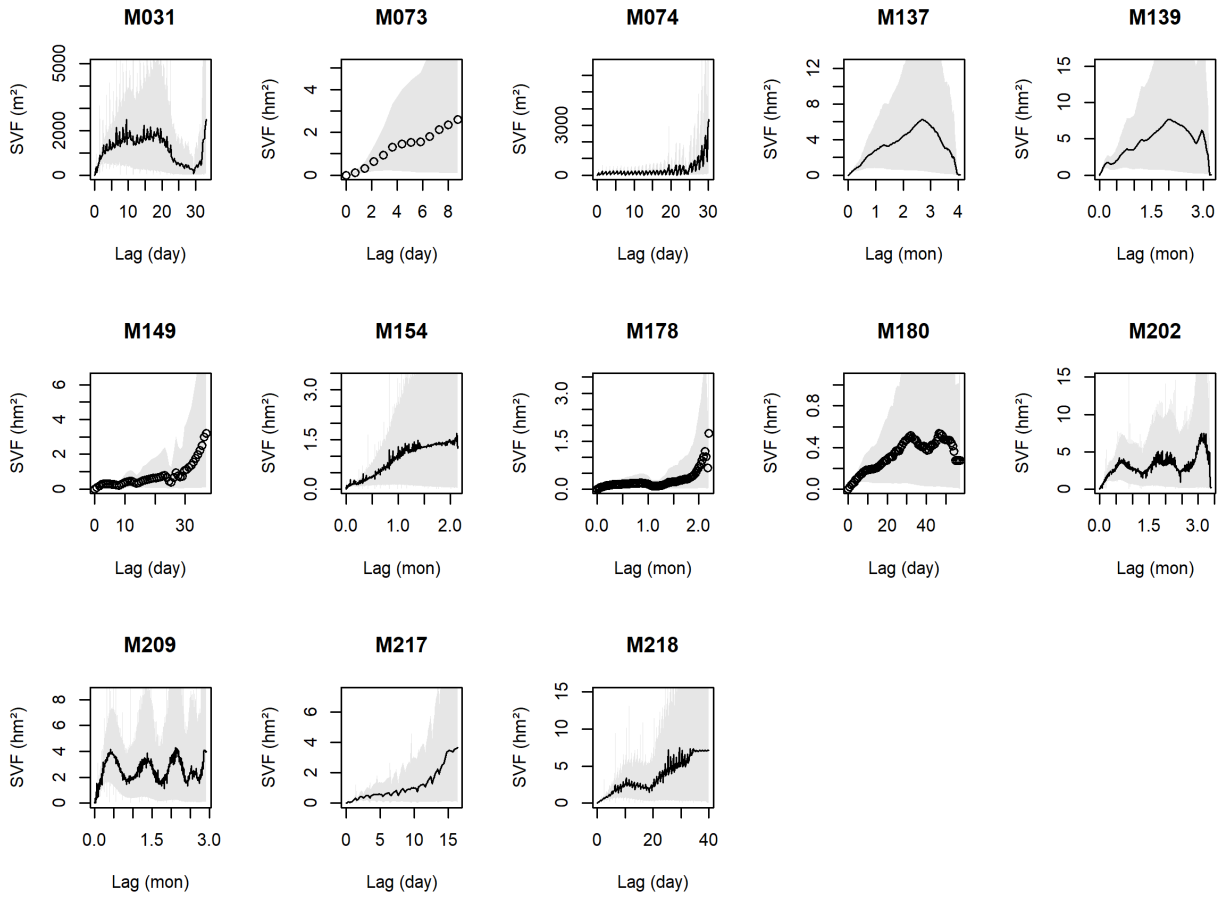

Figure S2.4: Variogram plots visualising the semi-variance function for tracked male snakes. M031, M137, M139, M154, M180, M202, M209 and M218 can be said to have reached range residency as the plots reach an asymptote and stop rising.

Table S2.2: Model fitting and selection results from the AKDE home range analysis. Model abbreviations are as follows: OU = Ornstein-Uhlenbeck, OUF = Ornstein-Uhlenbeck foraging process, IID = independently and identically distributed. dRMSPE is the root mean squared prediction error. DOF area is the effective sample size.

ID	movMod	dAICc	dRMSPE (m)	DOF area
F050	OU anisotropic	0.000	79.135	1.398
F050	OUF anisotropic	1.929	44.597	3.139

F050	OUf anisotropic	39.825	6.296	16.097
F050	OU	57.092	11.335	4.040
F050	OUF	59.052	6.893	8.309
F050	IID anisotropic	121.699	0.000	56.000
F142	OU anisotropic	0.000	30.813	1.857
F142	OUF anisotropic	1.548	22.051	2.429
F142	OUf anisotropic	5.364	3.333	16.888
F142	OU	14.431	12.961	3.708
F142	OUF	16.331	10.639	4.609
F142	IID anisotropic	59.757	0.000	56.000
F158	OU	0.000	11.896	8.719
F158	OUF	1.936	6.396	14.030
F158	OU anisotropic	2.750	11.884	8.806
F158	OUF anisotropic	4.777	6.425	14.116
F158	OUf	83.244	0.000	46.244
F158	OUf anisotropic	83.727	0.704	45.317
F158	IID	352.640	0.221	101.000
F159	OUF anisotropic	0.000	62.952	5.986
F159	OUF	3.888	92.126	5.695
F159	OUf anisotropic	32.321	0.000	24.656
F159	OU anisotropic	35.126	86.576	3.656
F177	OU anisotropic	0.000	1.425	35.567
F177	OUF anisotropic	2.106	1.049	41.296
F177	OU	5.475	1.306	36.449

F177	OUF	7.513	0.945	42.162
F177	OUf anisotropic	55.202	1.125	72.027
F177	IID anisotropic	226.211	0.000	125.000
F203	OU anisotropic	0.000	0.000	27.028
F203	OUF anisotropic	1.900	1.111	28.238
F203	OUf anisotropic	6.727	20.652	39.683
F203	IID anisotropic	59.275	65.023	65.000
F203	OU	66.136	379.251	17.983
F203	OUF	68.339	372.374	18.521
F212	OU anisotropic	0.000	15.803	2.006
F212	OUf anisotropic	0.391	0.000	7.678
F212	OUF anisotropic	1.609	9.021	2.977
F212	IID anisotropic	57.909	8.675	23.000
F212	OU	72.455	51.705	1.439
F212	OUF	73.870	41.980	1.738
F219	OU anisotropic	0.000	65.082	2.363
F219	OUF anisotropic	2.776	31.911	5.538
F219	OUf anisotropic	5.548	15.829	7.386
F219	IID anisotropic	21.476	0.000	25.000
F219	OU	34.915	193.764	1.562
F219	OUF	37.024	97.352	3.290
M031	OU anisotropic	0.000	4.935	19.441
M031	OUF anisotropic	2.009	2.932	25.168
M031	OU	65.621	7.557	18.805

M031	OUF	67.577	5.386	24.709
M031	OUf anisotropic	80.786	0.089	44.489
M031	IID anisotropic	485.599	0.000	164.000
M073	OUf anisotropic	0.000	29.358	9.967
M073	OU anisotropic	0.932	28.000	4.069
M073	OUF anisotropic	5.670	0.000	0.000
M073	IID anisotropic	22.338	24.694	17.000
M073	OUF	57.510	45.703	5.752
M073	OUf	65.971	29.656	10.357
M074	OUF anisotropic	0.000	3.331	15.079
M074	OU anisotropic	13.820	5.907	7.628
M074	OUf anisotropic	19.072	0.000	44.979
M074	OUF	166.274	2.059	58.139
M137	OU anisotropic	0.000	38.972	9.360
M137	OUF anisotropic	1.905	23.407	14.792
M137	OU	197.305	107.496	6.067
M137	OUF	199.126	79.049	10.967
M137	OUf anisotropic	274.591	0.000	85.081
M137	IID anisotropic	888.175	54.997	208.000
M139	OU anisotropic	0.000	44.243	8.691
M139	OUF anisotropic	1.924	26.855	13.932
M139	OUf anisotropic	145.798	0.000	52.998
M139	OU	251.646	139.605	5.271
M139	OUF	253.592	138.154	5.455

M139	IID anisotropic	550.045	78.689	166.000
M149	OU anisotropic	0.000	16.780	8.905
M149	OUF anisotropic	2.208	11.574	0.000
M149	OUf anisotropic	17.924	0.000	33.490
M149	OU	97.720	47.619	4.575
M149	OUF	99.381	39.930	5.686
M149	IID anisotropic	126.380	0.607	62.000
M154	OU anisotropic	0.000	22.310	14.270
M154	OUF anisotropic	1.913	16.506	20.202
M154	OU	67.108	33.923	12.579
M154	OUF	68.117	33.237	13.625
M154	OUf anisotropic	354.253	0.000	76.143
M154	IID anisotropic	1299.387	17.920	301.000
M178	OU anisotropic	0.000	41.954	9.502
M178	OUF anisotropic	2.000	35.395	15.055
M178	OUf anisotropic	60.893	19.416	39.676
M178	OU	132.452	10.928	18.604
M178	OUF	134.438	8.579	25.770
M178	IID anisotropic	165.861	0.000	108.000
M180	OU anisotropic	0.000	3.652	14.887
M180	OUF anisotropic	2.094	0.642	20.382
M180	OU	45.310	17.582	10.909
M180	OUF	47.282	12.577	16.344
M180	OUf anisotropic	53.742	0.000	49.021

M180	IID anisotropic	216.504	8.612	95.000
M202	OU anisotropic	0.000	42.097	18.359
M202	OUF anisotropic	1.911	31.826	24.517
M202	OU	35.568	52.740	17.494
M202	OUF	37.459	41.627	23.764
M202	OUf anisotropic	851.468	0.000	127.852
M202	IID anisotropic	1790.522	24.339	456.000
M209	OUF anisotropic	0.000	38.160	20.562
M209	OU anisotropic	55.661	41.911	14.858
M209	OUF	67.018	56.547	19.221
M209	OUf anisotropic	339.884	0.000	136.143
M217	OUF anisotropic	0.000	103.607	1.837
M217	OU anisotropic	2.957	128.102	1.582
M217	OUF	36.950	190.779	1.589
M217	OUf anisotropic	57.720	0.000	17.417
M218	OU anisotropic	0.000	165.379	2.623
M218	OUF anisotropic	2.066	164.682	2.652
M218	OU	45.480	94.254	3.521
M218	OUF	47.235	49.683	7.543
M218	OUf anisotropic	251.001	37.463	47.286
M218	IID anisotropic	1171.752	0.000	191.000

Chapter 3: Supplementary material

Table S3.1: Details of genetic samples used in this study.

Sample	Genus	Species	Locality	Country	Latitude	Longitude	Sex	Type
z106	*Zamenis*	*longissimus*	Bressanone, Bolzano (Trentino-Alto Adige)	Italy	46.78	11.64	unknown	scale clip
z34	*Zamenis*	*longissimus*	Farfanara, Parma (Emilia-Romagna)	Italy	44.84	9.98	unknown	tissue
z80	*Zamenis*	*longissimus*	Canepa, Repubblica San Marino	Italy	43.93	12.44	unknown	tissue
z28	*Zamenis*	*longissimus*	Castel Fusano, Roma (Latium)	Italy	41.73	12.32	unknown	scale clip
z92	*Zamenis*	*longissimus*	Fiamignano, Rieti (Latium)	Italy	42.26	13.13	unknown	tissue
z93	*Zamenis*	*longissimus*	Varco Sabino, Rieti (Latium)	Italy	42.24	13.02	unknown	tissue
4026	*Zamenis*	*longissimus*	Le Grand Momesson, Bouvron	France	47.40	-1.88	adult male	scale clip
4029	*Zamenis*	*longissimus*	Le Grand Momesson, Bouvron	France	47.40	-1.88	subadult female	scale clip
4042	*Zamenis*	*longissimus*	Malville	France	47.47	-1.87	adult male	scale clip
4043	*Zamenis*	*longissimus*	La Fraudais, Blain	France	47.50	-1.74	subadult male	scale clip
4044	*Zamenis*	*longissimus*	La Fraudais, Blain	France	47.50	-1.74	adult male	scale clip
4045	*Zamenis*	*longissimus*	La Gandonnais	France	47.39	-1.86	adult female	scale clip

1549	*Zamenis*	*longissimus*	Colwyn Bay	Wales	53.29	-3.75	unknown	liver
1550	*Zamenis*	*longissimus*	Colwyn Bay	Wales	53.30	-3.75	unknown	liver
1773	*Zamenis*	*longissimus*	Colwyn Bay	Wales	53.29	-3.75	unknown	scale clip
w63	*Zamenis*	*longissimus*	Colwyn Bay	Wales	53.29	-3.74	adult female	scale clip
w94	*Zamenis*	*longissimus*	Colwyn Bay	Wales	53.29	-3.74	adult male	scale clip
w137	*Zamenis*	*longissimus*	Colwyn Bay	Wales	53.30	-3.75	adult male	scale clip
3110	*Zamenis*	*situla*	Captive specimen, UK				unknown	shed skin
XT264/17	*Zamenis*	*longissimus*	London Zoo	England	51.54	-0.16	adult male	tissue
XT1098/17	*Zamenis*	*longissimus*	London Zoo	England	51.54	-0.15	juvenile unknown	tissue
XT673/18	*Zamenis*	*longissimus*	London Zoo	England	51.54	-0.16	juvenile male	tissue
XT901/18	*Zamenis*	*longissimus*	London Zoo	England	51.54	-0.15	adult female	tissue
XT450/20	*Zamenis*	*longissimus*	London Zoo	England	51.54	-0.16	adult female	tissue
A3	*Zamenis*	*longissimus*	Bretzenheim	Germany	49.88	7.89	unknown	tissue
B	*Zamenis*	*longissimus*	Eltville/Taunus	Germany	50.00	8.00	unknown	shed skin
E2	*Zamenis*	*longissimus*	Passau/Donau	Germany	48.58	13.44	unknown	shed skin
RM5419	*Zamenis*	*longissimus*	Near Iasi, NE Romania	Romania	47.03	27.58	unknown	tissue
RM5419	*Zamenis*	*longissimus*	Near Iasi, NE Romania	Romania	47.03	27.58	unknown	tissue

Table S3.2: Summary statistics from the trimming, merging, and mapping of resequenced samples.

Sample	Country	Raw reads	Reads merged	Coverage (mean depth)	Mapped bp	Mapped Gb	Merged duplication rate
A3	Germany	59800741	2326903	2.47	3150484941	3.15	0.15
B	Germany	80046913	19747878	11.13	16763417299	16.76	0.16
E2	Germany	103311154	55846325	9.66	15035010140	15.04	0.16
Fr4026	France	41951567	21608127	2.36	2908723187	2.91	0.13
Fr4029	France	44942564	21750275	5.02	7619894257	7.62	0.16
Fr4042	France	55617588	26911354	6.34	9401873401	9.40	0.17
FR4043	France	39572853	22332689	4.37	6377898207	6.38	0.16
Fr4044	France	45370659	24869370	4.9	7144192481	7.14	0.18
Fr4045	France	41045955	19240835	4.49	6740995828	6.74	0.17
RM5419	Romania	97917067	31838008	13.71	20616425591	20.62	0.15
RM5420	Romania	83327754	23142762	11.78	17709534612	17.71	0.15
s1549	Wales	26391404	23035791	2.82	3529908477	3.53	0.13
s1550	Wales	51568132	33760392	5.35	7855735276	7.86	0.17
s1773	Wales	34528584	18018143	4.1	5984471404	5.98	0.14
W108	Wales	132379474	37106651	51.45	82795184752	82.80	0.18
W137	Wales	48920374	20872513	5.58	8292333893	8.29	0.20
W63	Wales	50986478	23306772	5.67	8698886171	8.70	0.19
W94	Wales	49301666	24850577	5.51	8198954471	8.20	0.18
XT264	London	29835662	25970684	3.05	3931292450	3.93	0.12
XT450	London	37150999	28218712	1.66	1424768609	1.42	
XT673	London	27597734	25450396	2.28	2692974038	2.69	0.10
XT901	London	35787137	31640101	2.83	3730899292	3.73	0.14
Z28	Italy	34873310	19261299	4.14	6005715993	6.01	0.14
Z80	Italy	32746971	21706860	3.63	5127865533	5.13	0.13
Z92	Italy	47788570	32929152	4.47	6615320039	6.62	0.20
Z93	Italy	47092626	28030972	4.79	7014151275	7.01	0.20
Z3110	Captive *Zamenis situla*	43069335	40277014	3.2	3829757996	3.83	0.15

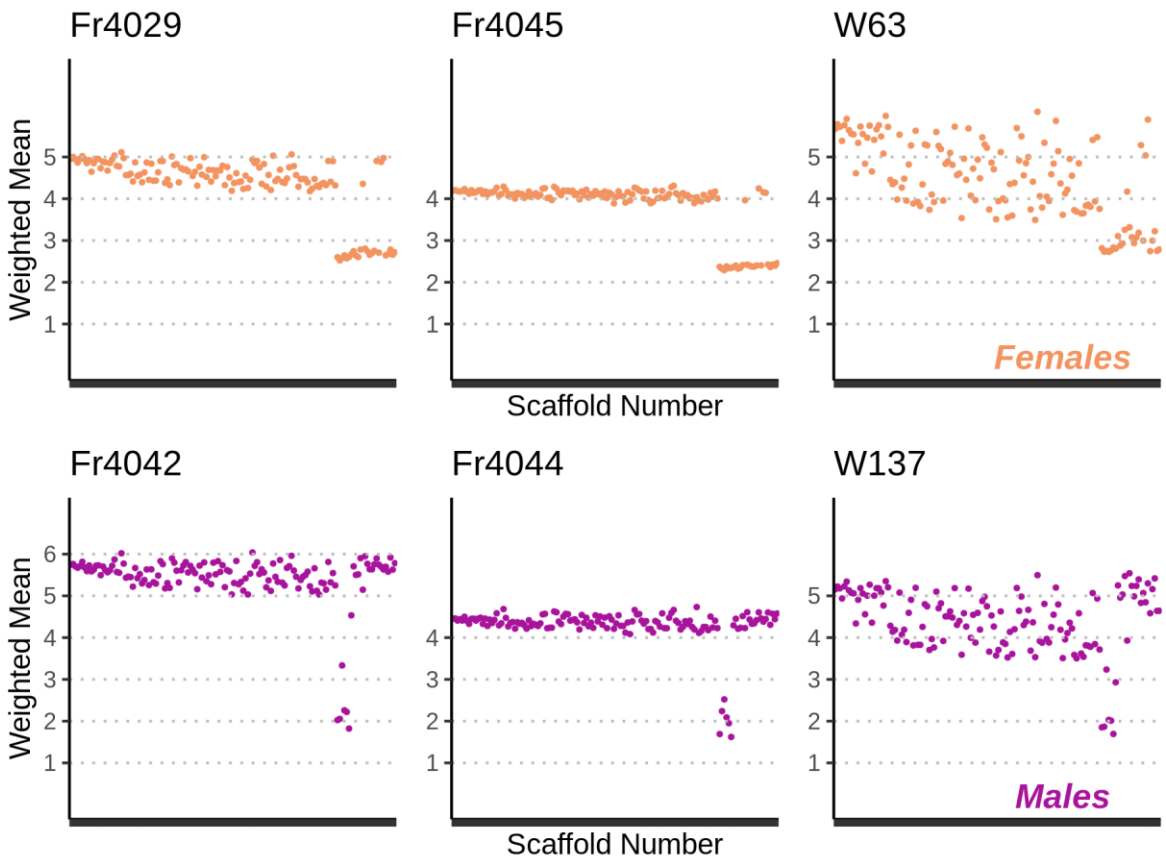

Figure S3.1: Plots of six individual snakes showing the weighted mean depth of coverage of each genome scaffold over 1 Mb in length. Each dot represents one scaffold. Sex chromosomes can be identified by their reduced coverage.

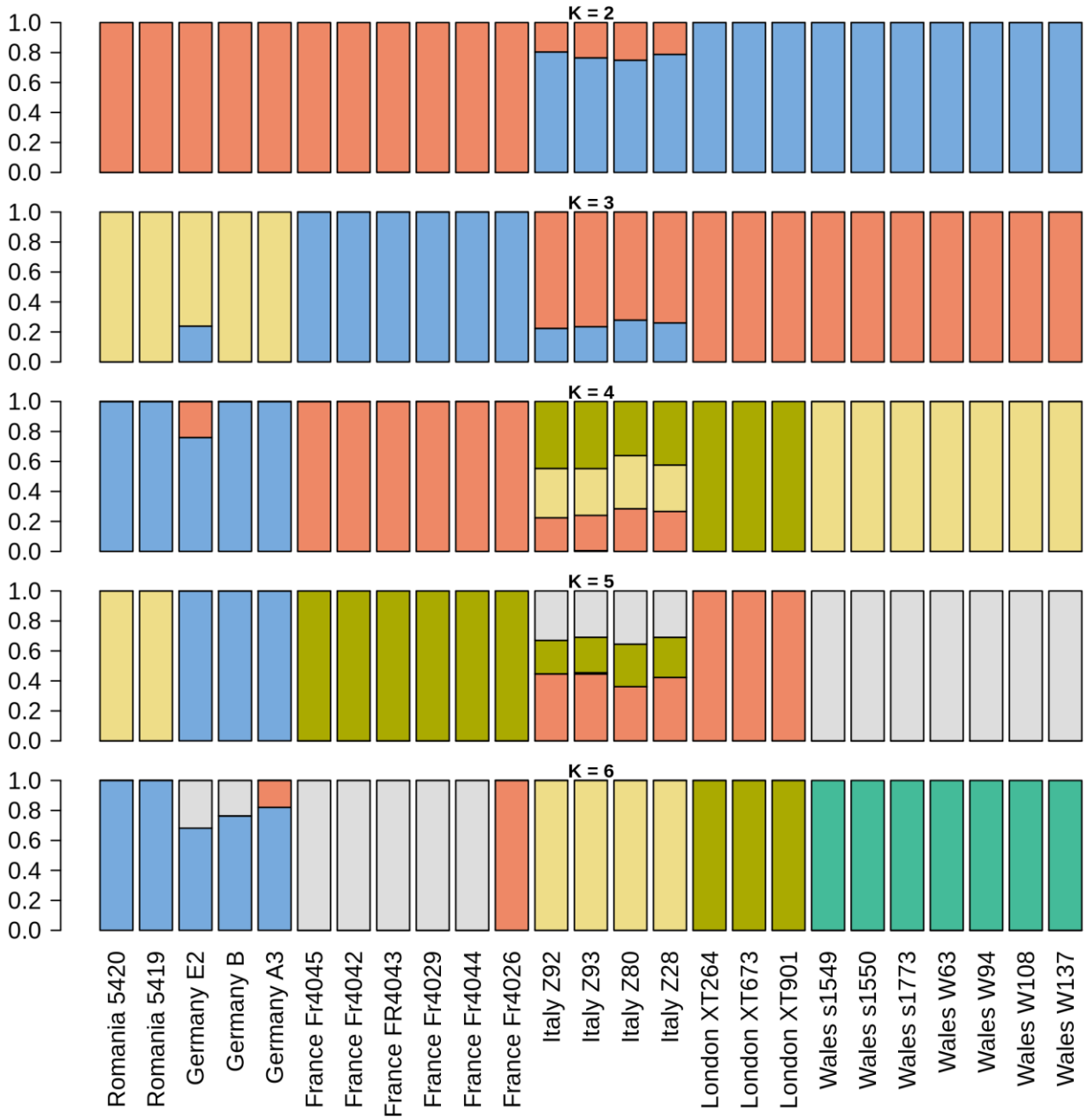

Figure S3.2: Admixture runs with a different seed. Analysis performed with NGSAdmix. Colours represent the different ancestral populations, the number of which is denoted by K.

Chapter 4: Supplementary material

Table S4.1: PCR program for 12S (Goebel et al., 1999)

Step	Thermocycler	Temperature (°C)	Time
1	Initial denature	96	2 mins
2	Annealing	52	45 secs
3	Extension	72	2 mins
4	Denaturation	94	30 secs
5	Annealing	52	45 secs
6	Extension	72	90 secs
	Repeat 4-6 x 39		
7	Extension	72	10 mins
8	Cooling	4	10 mins

Table S4.2: PCR program for 16S (Palumbi, 1996)

Step	Thermocycler	Temperature (°C)	Time
1	Initial denature	94	2 mins
2	Denature	94	30 secs
3	Annealing	50	30 secs
4	Extension	72	45
	Repeat 2 - 4 x 35		
5	Final extension	72	5 mins
6	Cooling	4	10 mins

Table S4.3: Morphological data used in the *Hemachatus* morphometric analysis.

Museum_nu	Locality	State	Group	SVL	Tail_length	Total_length	Sex	Nape_scale_r	Midbody_sca
UM31402	Troutbeck,	Zimbabwe	zim	490	97	587	2	17	17
UM31317	Charles	Zimbabwe	zim	142	26	168	1	16	17
UM3061	Mare Dah,	Zimbabwe	zim	578	102	680	2	17	17
UM23767	Marika,	Zimbabwe	zim	560	125	685	1	17	19
UM16098	Inyanga	Zimbabwe	zim	865	150	1015	2	17	19
UM13413	Manyoli,	Zimbabwe	zim	206	39	245	2	17	19
UM1307	5 km NW of	Zimbabwe	zim	635	133	768	1	18	19
UM11848	Mare Dah,	Zimbabwe	zim	140	22	162	2	17	19
UM11847	Mare Dah,	Zimbabwe	zim	270	55	325	1	17	18
Paget.Coll.	Charles	Zimbabwe	zim	615	127	742	1	17	17
NMZB9503	Trout	Zimbabwe	zim	625	121	746	2	17	17
NMSR5121	Inyanga	Zimbabwe	zim	NA	NA	NA	NA	NA	17
NMSR5120	Nyamaziwa,	Zimbabwe	zim	NA	NA	NA	NA	NA	NA
NMSR5119	Nyamaziwa,	Zimbabwe	zim	NA	NA	NA	NA	NA	17
UM9632	Lothair	Transvaal	other	650	150	800	1	17	17
UM9631	Port	Cape	other	NA	NA	NA	2	17	19
UM5860	Port	Cape	other	NA	NA	NA	1	17	19
UM12027	Port	Cape	other	NA	NA	NA	1	17	19
NMZB-	Polela	Natal	other	170	26	196	NA	19	19
NMZB-	South Africa'	Orange Free	other	155	31	186	NA	17	18
NMZB979	Eastern Cape	Cape	other	1000	210	1210	1	19	19
NMZB7066	Johannesburg	Transvaal	other	1080	185	1265	2	17	17
NMZB5553	?	Cape	other	750	165	915	1	19	19
NMZB17611	Standord,	Cape	other	590	130	720	1	19	19
NMSR3892	Port	Cape	other	NA	NA	NA	1	19	19
NMSR1963	Johannesburg	Transvaal	other	NA	NA	NA	2	17	19
BM.62.9.2.26	Cape of Good	Cape	other	NA	NA	NA	2	NA	NA
BM.55.10.16.	South Africa	Cape	other	NA	NA	NA	1	NA	NA
BM.55.10.16.	South Africa	Cape	other	NA	NA	NA	1	NA	NA
BM.55.10.16	South Africa	Cape	other	NA	NA	NA	1	NA	NA
BM.45.2.21.1	South Africa	Cape	other	NA	NA	NA	2	NA	NA
BM.11.7.1a	Cape of Good	Cape	other	NA	NA	NA	1	NA	NA
AMNH51839	Big Tugela	Natal	other	NA	NA	NA	1	19	19
AMNH51838	Kroonstad	Orange Free	other	940	200	1140	1	17	19
2415	Uilenkraal,	Western Cape	other	328	76	404	1	NA	NA
2413	Uilenkraal,	Western Cape	other	688	142	830	2	NA	NA

Pre-cloacal_scale_rows	Ventrals	Cloacal	Sub-caudals	Supralabials	Upper_labials	Lower_labial	In_contact_an	Preoculars	Postoculars
11	128	E	33	7	3,4	8	4	1	3
13	119	E	34	7	3,4	8	4	1	3
13	126	E	35	7	3/3,4	8	4	1	3
13	124	E	38	7	3,4	8	4	1	3
13	128	E	34	7	3,4	8	4	1	3
13	130	E	37	7	3,4	8	4	1	3
13	123	E	38	7	3,4	8	4	1	3
13	126	E	30	7	3,4	8	4	1	3
13	122	E	35	6.5	3,4	8	4	1	3
13	122	E	37	7	3,4	7.5	3.5	1	3
13	130	E	34	7	3,4	8	4	1	3
NA	NA	NA	NA	7	3,4	8	4	1	3
NA	NA	NA	NA	7	3,4	8	4	1	3
NA	NA	NA	NA	7	3,4	8	4	1	3
13	130	E	41	7	3,4	8	4	1	3
13	148	E	40	7	3,4	9	4	1	3
13	133	E	38	7	3,4	8	4	1	3
13	134	E	44	7	3,4	8.5	4	1	3
13	NA	E	37	7	3,4	8	4	1	3
13	NA	E	43	6	3	8	4	1	3
13	131	E	43	7	3,4	8	4	1	3
13	143	E	38	7	3,4	7.5	4	1	3
13	135	E	42	7	3,4	8	4	1	3
13	127	E	42	7	3,4	8	3.5	1	3
14	134	E	43	7	3,4	8	4	1	3
13	145	E	43	7	3,4	8	4	1	3
NA	140	NA	39	NA	NA	NA	NA	NA	NA
NA	121	NA	35	NA	NA	NA	NA	NA	NA
NA	118	NA	38	NA	NA	NA	NA	NA	NA
NA	117	NA	37	NA	NA	NA	NA	NA	NA
NA	129	NA	35	NA	NA	NA	NA	NA	NA
NA	120	NA	39	NA	NA	NA	NA	NA	NA
13	138	E	44	7	3,4	NA	NA	1	3
13	134	E	46	7	3,4	NA	NA	1	3
NA	NA	NA	39	NA	NA	NA	NA	NA	NA
NA	NA	NA	39	NA	NA	NA	NA	NA	NA

Temporals	x	y
2+3/2+2	32	18
2+3	32	18
2+3	32	18
2+3	32	18
2+3	32	18
2+2/2+3	32	18
2+2/2+3	32	18
2+3	32	18
2+3	32	18
2+3	32	18
2+3	32	18
2+3	32	18
2+3	32	18
2+2/2+3	32	18
2+3	30.4	-26.3
2+4/2+3	25	-33
2+3	25	-33
2+3	25	-33
2+3	29.7	-29.8
2+3	NA	NA
2+3	NA	NA
2+3	NA	NA
2+3	NA	NA
2+3	NA	NA
2+3	NA	NA
2+3/2+5	25	-33
2+3	NA	NA
2+3	19	-34
2+3	NA	NA
2+3	NA	NA
2+3	NA	NA
2+3	NA	NA
NA	NA	NA
NA	NA	NA
NA	NA	NA
NA	NA	NA
NA	NA	NA
NA	NA	NA
NA	NA	NA
2+3	27.2	-27.6
2+3	30	-28
NA	18.4	-34.3
NA	NA	NA
NA	NA	NA
NA	19.47	-34.56
NA	19.47	-34.56

Table S4.4: Samples and GenBank accession numbers for specimens used in the genetic analysis.

Species	Description	12S	16S	Cytochrome b	NADH 4
Aspidelaps scutatus	-	U96790	KX694617	AF217828	AY058969
***Hemachatus inyangae* sp. nov.**	NMZB 9503 Nyanga, Zimbabwe	OQ857311	OQ857301	-	-
Hemachatus haemachatus	2337 KwaZulu-Natal	OQ857312	OQ857302	MT346639	MT346831
Hemachatus haemachatus	2658 KwaZulu-Natal	OQ857313	OQ857303	-	-
Hemachatus haemachatus	2263 Eswatini	OQ857314	OQ857304	MT346634	MT346826

Hemachatus haemachatus	2361 Western Cape	OQ857315	OQ857305	MT346641	MT346833
Hemachatus haemachatus	2596 Eastern Cape	OQ857316	OQ857306	-	-
Hemachatus haemachatus	2338 KwaZulu-Natal	OQ857317	OQ857307	MT346640	MT346832
Hemachatus haemachatus	2320 Gauteng	OQ857318	OQ857308	MT346638	MT346830
Naja nigricollis	1074	EU624237	GQ359754	GQ359505	AY713377
Naja ashei	1430 Watamu, Kenya	GQ359656	GQ359742	GQ359493	GQ359575
Naja mossambica	190 (12S, cytb), 1391 (ND4)	GQ359658	GQ359744	MT346654	MT346851
Naja katiensis	1540 Doussoudiana, Mali	GQ359657	GQ359743	GQ359494	GQ359576
Naja pallida	1080 Tanzania	GQ359659	GQ359745	GQ359496	GQ359578
Naja nubiae	837 (12S, 16S) 4565	GQ359660	GQ359746	MT346686	MT346881

	(cytb, ND4)				
Naja nivea	1295	EU624238	GQ359755	MT346759	MT346935
Naja arabica	1681	GQ359663	GQ359749	GQ359500	GQ387077
Naja haje	Egypt 893	GQ359664	GQ359750	GQ359501	GQ387063
Naja haje	Kenya 1262	GQ359661	GQ359747	GQ359498	GQ359580
Naja senegalensis	Senegal 2203 (cytb, ND4), Mali 1542 (12S, 16s)	GQ359666.	GQ359752	MT346763.	GQ387080
Naja annulifera	881	GQ359667	GQ359753	GQ387119	GQ387090
Naja annulata	-	U96792	AY188049	MT346699	MT346895
Naja naja	ABTC32465	EU547088	EU547137	EU547039	EU546997
Naja naja	579	EU624236	GQ359756	MT346711	MT346907
Naja atra	582 (Hong Kong - cytb, ND4), mitogenome (12S, 16S)	EU921898	EU921898	MT346703	MT346899
Naja kaouthia	812 Southern Vietnam	LC431744	LC431744	LC431744	LC431744

Naja sputatrix	584 Java, Indonesia	OQ857319	OQ857309	MT346730	MT346922
Naja sumatrana	586 (ND4, cytb)	JN687928	JN687929	MT346735	MT346931
Naja siamensis	-	JN687926	JN687927	MT346726	MT346924
Ophiophagus hannah	China	EU921899	EU921899	EU921899	EU921899
Ophiophagus hannah	Indonesia	AZIM010092 53	AZIM010092 53	AZIM010092 53	AZIM010092 53
Pseudohaje goldii	1336	OQ857320	OQ857310	MT346778	MT346952
Walterinnesia aegyptia	-	U96807	HQ267785	MT346780	AY058988

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Additional publication: Marking the un-markable: visible implant elastomer in wild juvenile snakes

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Published in *The Herpetological Journal* (2020): https://doi.org/10.33256/hj30.3.173176

Abstract

Marking individuals is a key component of many ecological studies, but with some animals, such as juvenile snakes, it has proven problematic because of size constraints. This impedes our understanding of their habits in the wild. We marked juvenile Aesculapian snakes (*Zamenis longissimus*) in North Wales with visible implant elastomer (VIE), and recaptured them the following season. Our results demonstrate that the use of VIE is an effective marking method for small snakes, negating the need for tissue removal when marking. We suggest it represents a promising development in the ecological study of snakes, and is especially useful in species that undergo ontogenetic pattern changes.

Capture-mark-recapture (CMR) studies are a key method in ecology to collect data on birth rates, death rates, movement patterns, and abundance of individuals (Krebs, 1989). As most CMR studies rely on the identification of one individual from another, unique marks or patterns are key to an effective study. The marks must meet several fundamental criteria: the mark cannot be lost; it must not affect the survival of the individual; it must not affect the likelihood of recapture; and it must be recordable (Otis et al., 1978). Because of their small size, juvenile snakes have been among the most difficult animals to mark (Winne et al., 2006). This has been a contributing factor to the ecology and dispersal behaviours of juvenile snakes being little-known (Ferner & Plummer, 2016).

There are many different methods by which a mark can be applied to snakes (Haines & Modde, 1996; Powell & Proulx, 2003). Externally mounted tags can be shed or knocked off when the snake is active and may hinder the snake in its movement. Ventral clipping marks are less obtrusive to the animal, but can sometimes be confused due to new scars on the ventral side of the snake, and substantial regrowth of clipped tissue. The same is true for marks made with cauterising units.

Passive integrated transponders (PIT) tags are the most reliable, albeit most expensive, method of identifying individual snakes. However, due to their size, PIT tags are unsuitable for use in smaller snakes.

It is possible in some cases to use body colour and pattern to identify individual snakes. However, both are susceptible to ontogenic change in many species, where the pattern is altered or altogether lost as the individual ages (Creer, 2005). Head scalation is also used, and while changes in scalation are not common, ontogenic changes have been documented in *Vipera Ursinii* (Tomović et al., 2008), and changes due to injury have been seen in *Vipera berus* (Bauwens, Claus & Mergeay, 2018). In the case of the Aesculapian snake (*Zamenis longissimus*), as juveniles the snakes sport a distinct black and yellow chequered chin and ventral pattern which is unique to that individual. As the individual grows, the chin and ventral patterns are completely lost to a homogenous olive-green or brown colour along the flanks and dorsum of the body, and a solid yellow colour on the ventral scales. Without continuous observation of the individuals, and, currently, a reliable marking method for juvenile snakes, it is therefore very challenging to identify an adult from their juvenile form.

In this study we present a novel method for marking wild-caught, juvenile snakes, by application of visible implant elastomer (VIE). VIE is an inert, biocompatible polymer. The elastomer is injected as a liquid which cures into a pliable solid under the skin of the individual. The marks fluoresce under UV-B light and are externally visible. Whilst VIE has been criticised as unreliable in frogs (Brannelly, Chatfield & Richards-zawacki, 2013), it has successfully been used in many different animal species including turtles (Anderson et al., 2015), lizards (Schmidt & Schwarzkopf, 2010), and conversely, frogs (Sapsford et al., 2014; Bainbridge et al., 2015). This method has also been used in salamanders, including the Eastern red-backed salamander (Heemeyer, Homyack & Haas, 2007) and the Northern two-lined salamander (Bailey, 2004). Northern two-lined salamanders are small and slender, making them difficult to mark, similar to juvenile snakes. With regard to snakes, (Hutchens et al., 2008) successfully implanted 18 corn snakes (*Pantherophis guttutas*), and showed the marks lasted over a year under lab conditions, but there are no published studies that look at VIE as a viable method of marking wild-caught snakes. Here, we demonstrate the use of VIE as a simple, effective method for marking small wild snakes for use in CMR studies.

This study took place as part of a CMR study of introduced Aesculapian snakes (*Zamenis longissimus*) in Colwyn Bay, North Wales. A mark scheme was generated using software Salamarker (The Williams Lab, Purdue University; (MacNeil, Dharmarajan & Williams, 2011).

Twelve marking locations and two colours produced 264 unique mark combinations. A Visible Implant Elastomer Manual Injections Kit (Northwest Marine Technology, Inc) was used to mark the animals.

We marked wild juvenile snakes (snakes that weighed under 40g, and likely under three years old) (N = 43) with two elastomer marks each from 21 April to 17 October 2018, using either fluorescent red or fluorescent yellow VIE, or one of each. We selected these colours to contrast with the dark base colour of the Aesculapian snake. The injection was made using a 29-gauge needle in the interstitial skin. A fold of loose skin was made by gently pinching the skin together dorsolaterally, creating a pocket for the needle to enter. The needle was directed anteriorly, and approximately 0.02 ml were injected at each location, in a small 'stripe' three scale rows in length. After application the marks were checked for external visibility under UV-B light. The snakes were then released at their capture site.

Snakes caught in the following season (2019) were checked for VIE marks. Snakes were also compared to a record of head and chin images to ensure any snakes that may have shed their marks and, that any movement of marks did not affect the ability to ID individuals. In 2019 seven individuals were recaptured with elastomer marks. The snakes were successfully identified using the position of the VIE marks along the body in accordance with the mark scheme. The snakes' identities were confirmed using photos of the individuals' unique chin pattern and head scalation. In the 2019 season all juvenile unmarked snakes (N=27) were new to the study and there was no evidence of mark loss in any of the snakes, this was confirmed against photos of head scalation. Snakes were recaptured an average of 377±36 days after their initial capture (range 306 – 434). Both red and yellow marks were easily recognisable using a UV-B light, under which the marks fluoresce (Fig. 1).

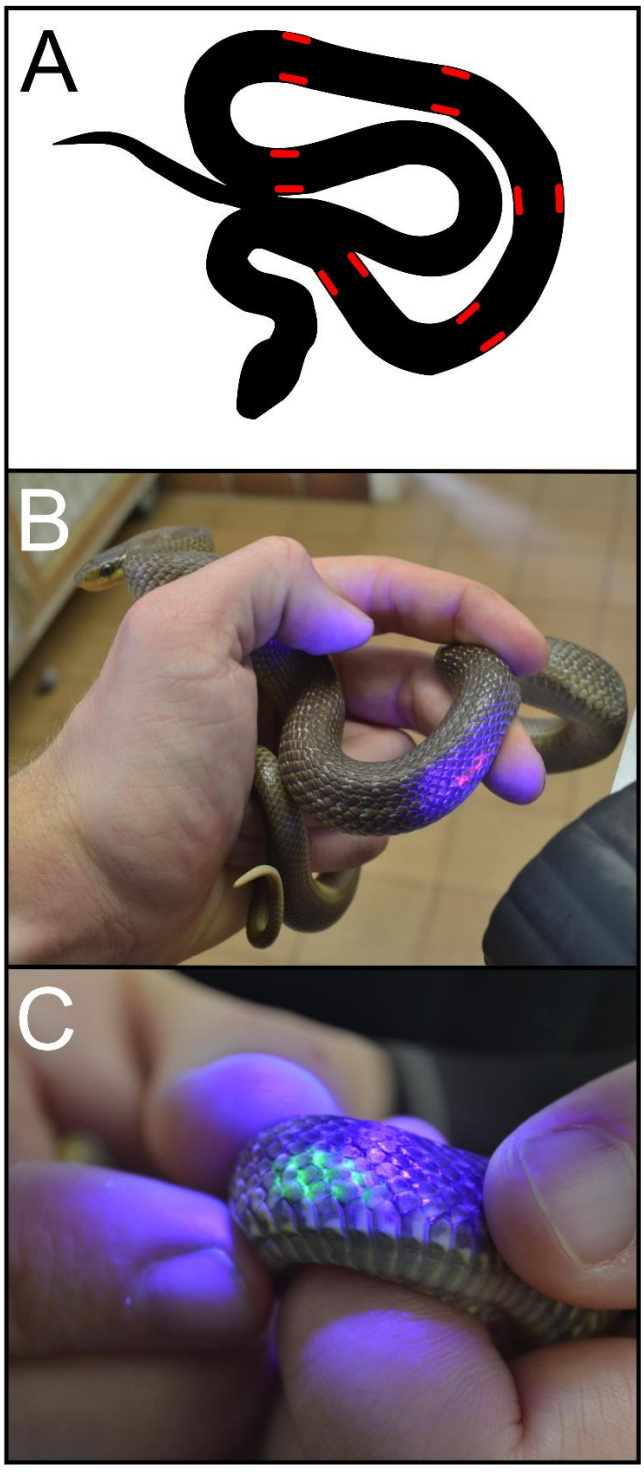

Fig. 1. Examples of visible implant elastomer used in wild Aesculapian snakes; A) the approximate locations of the mark zones, with 12 possible locations in the elastomer mark scheme; B) the red mark shown under UV-B light 381 days after initial insertion (ZALO049); C) close up of yellow mark under UV-B 390 days after insertion (ZALO042)

Upon initial capture in 2018, the seven juvenile snakes which went on to be recaptured averaged 299±58 mm SVL (range 244 – 407 mm), and 367±72 mm (range 327 – 625 mm) total length (TL). The average mass was 16.14±9.26 g (range 8.52 – 32.26 g). When recaptured during the 2019 season, the snakes averaged 392±98 mm SVL (range 267 – 525 mm), and 469±112 mm TL (range 327 – 625 mm). The mass of recaptured snakes averaged 28.64±23.37 g (range 11.89 – 49.96 g). All the snakes recaptured the following year gained weight, with the mean mass gain being 61±43 % (range 12 – 139 %).

All of the marks retained high external visibility under UV-B light. Of the 14 marks applied to recaptured snakes, 13 remained intact at their initial application site. In one instance, however, the mark stretched dorsolaterally, both anterior and posterior of the mark site, for a total of 30 scale rows. The mark was thickest at the original application site and became thinner as the elastomer travelled. We believe this to be a result of overapplication of the elastomer leading to dispersion prior to setting. While the mark was still decipherable, minimising the amount applied should avoid such complications.

In terms of the cost of VIE, a 6 ml kit costs £250 and will mark approximately 80 snakes, with refill packs costing less than £100. To compare this with PIT tags, the cost will be around £230 for the reader and the tags, with the PIT tags costing around £130 for 50 snakes. The VIE kit is more expensive in terms of initial cost, however once the cost of restocking is considered, VIE is cheaper than the PIT tags per snake. However, it is worth noting that VIE will be more costly when marking larger snakes, as larger amounts will have to be used to effectively mark individual snakes.

Here we present the first evidence of long-term reliability of VIE tags in wild snakes. This marking method will aid in addressing major knowledge gaps in the ecology of small, slender, and juvenile snakes that were previously impossible or difficult to mark. These species can now be marked reliably, greatly enhancing the possibilities for future ecological studies. We believe the evidence presented justifies the use of VIE in wild snakes, especially juveniles too small for PIT tags, and those which undergo dramatic ontogenetic change. Further work with both marked and unmarked snakes will better clarify the effects on snake fitness and survival.

Acknowledgements

This study was supported by KESS II and the Welsh Mountain Zoo. We gratefully thank the staff of the Welsh Mountain Zoo for their continued support and assistance.

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Additional publication: Mate today, gone tomorrow: male on female cannibalism in a wild Aesculapian snake (*Zamenis longissimus*) in North Wales

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Published in *Herpetology Notes* (2023).

Cannibalism is widespread in animals, but the consumption of adult females by adult males is considered rare and difficult to explain because eating an adult female represents the loss of a potential mate (Glaudas and Fento, 2021). Because of the secretive nature of snakes, and the paucity of information on the specific dietary requirements of many species, this phenomenon may be truly rare or simply under-reported, making observations of male-female cannibalism particularly valuable (Polis, 1981).

The Aesculapian snake *Zamenis longissimus* Laurenti, 1768, is a colubrid snake that lives in mainland Europe, from eastern Spain to Azerbaijan (Geniez, 2018). Sexual dimorphism is present, with males reaching up to 200 cm total length, with a long tail, and females reaching 140 cm total length, with shorter tails (Corti et al, 2011; Kreiner, 2007). The species is mainly diurnal but may become crepuscular during the hottest months in summer (Kreiner, 2007). It lives predominantly in mixed forests (Gomille, 2002), with lowland populations preferring more humid microhabitats, and highland populations preferring drier and more sun-exposed habitats (Corti et al, 2011). Aesculapian snakes are cryptic, making them difficult to spot even when basking (Kreiner, 2007). Females lay eggs in late summer, often in communal sites. These sites can be used by multiple species, such as *Natrix helvetica* and *Hierophis viridiflavus* (Corti et al, 2011).

The Aesculapian snake is considered a generalist species, but although they incorporate a wide variety of prey into their diet (Capula and Luiselli 2002), they are not known to be snake-eaters. This species displays an ontogenetic shift in diet; neonates and juveniles feed mainly on lizards and juvenile micromammals (e.g. voles, mice, shrews), and occasionally on amphibians and

invertebrates (Naulleau and Bonnet 1995, Najbar 2007, Lelièvre et al. 2012). In adults, most of the diet consists of terrestrial micromammals, birds, and their eggs. Some cases of chiropterophagy by adult Aesculapian snakes have been reported (Barti et al,. 2019).

The observation described here occurred as part of a radiotelemetry study on a population of Aesculapian snakes in Colwyn Bay, North Wales. This population stems from an accidental introduction in the 1970s and is outside the species' modern natural range. In 2021 we tracked nine adult snakes as part of our study investigating the movement patterns, space use and habitat use of this population.

M137 is an adult male (Fig 1B) captured on 10th May 2021 after being found beneath a black tarpaulin in the garden of a local resident. He weighed 512 g and measured 1178 mm snout-vent length (SVL) with a tail length of 280 mm. A radio transmitter was implanted on 1st June 2021 and the snake was released on 2nd June 2021.

F159, an adult female Aesculapian snake (Fig 1A), was initially captured on 11th June 2021 while basking in a hedgerow. At the time of capture, she weighed 266 g and measured 845 mm SVL with a tail length of 173 mm. Transmitter insertion was performed on 17th June 2021 and the snake was released on 18th June 2021.

Both snakes were fitted with Holohil BD2-T transmitters, with F159 receiving a 1.6g transmitter and M137 a 1.9 g transmitter. Transmitters were implanted following Reinert and Cundall (1982), with isofluorane anaesthesia and butorphanol analgesic. Following release, the snakes were tracked twice daily throughout the active season, at approximately 10:00 and 14:00.

From 17th August 2021 both snakes were suspected to be in a similar area, in or close to the grounds of an old country house. However, by this time the battery of F159's transmitter was running out, making it difficult to pinpoint her exact location. On 8th September 2021 during regular tracking, both M137 and F159 seemed to be inside a large rhododendron bush. RB spotted a snake inside the bush and caught it. The snake was discovered to be M137. The frequencies for both M137 and F159 were found to be coming from the same snake, with two radio transmitters inside M137. The only logical explanation was cannibalism – M137 had eaten F159. It is unclear whether F159 was predated or whether she died and the carcass was subsequently eaten. M137 was released on 8th September 2021.

The cannibalistic individual, M137, was recaptured the following Spring. On 19th June 2022 he was found to weigh 502 g and appeared to be in excellent body condition. We confirmed the female's radio transmitter was no longer inside his body using a magnetic stud finder. The female's transmitter had either passed through the gastrointestinal tract or was vomited up by the male snake - we believe it is unlikely that the transmitter ruptured through the body wall of M137 as there was no new scarring on the body.

Ophiophagy, the consumption of snakes as prey items, is a widespread trait among serpents and is found in species of most snake families (Jackson et al. 2004). From an energetic point of view, snakes as prey items represent a significant resource to other snakes, allowing them to ingest elongated prey and thus overcome their mouth gape constraint (Cundall and Greene, 2000; Wiseman et al., 2019). In fact, x-rays have shown that snakes are able to ingest prey exceeding their length, folding the prey's spine into waves within the gastrointestinal tract of the predator (Jackson et al. 2004).

What seems odd from an evolutionary perspective is the fact that the cannibalised individual was a female while the predator was a male. In fact, the presence of reproductive females is usually a limiting factor in most reproductive systems and feeding on a female could be perceived as damaging to future mating opportunities. Glaudas and Fuento (2022) highlighted the rarity of this kind of male-on-female cannibalistic event, discussing the potential ecological and evolutionary reasons behind this phenomenon. They stated that even if this behaviour could be seen as maladaptive, predation upon a conspecific female could provide males with significant energy intake, especially if females are not seen as a potential mate at the time of the predation. Shankar and Whitaker (2013) observed multiple adult male king cobras, *Ophiophagus hannah*, feeding on female conspecifics. They noted that this behaviour occurred outside the breeding season and hypothesised that hormones associated with mating suppress appetite in males during the breeding season, but they return to snake-eating, including cannibalism, later in the year.

Predominantly ophiophagic snakes are absent in Europe, but there are a few species, such as the European whipsnake, *Hierophis sp.,* the Montpellier snake, *Malpolon sp.*, and the smooth snake, *Coronella austriaca*, that are known to commonly feed on other snakes and even conspecifics (Safaei-Mahroo et al. 2017, Di Nicola et al. 2020, Jofré and Reading, 2020). This latter behaviour, cannibalism, has been historically perceived as odd and mostly confined to the captive environment (Jofré and Reading, 2020). Nevertheless, recent studies have highlighted a strong

underappreciation of this phenomenon, which is rarely observable, but may have significant ecological implications. For example, a recent investigation of the feeding habits of the cape cobra, *Naja nivea*, revealed that conspecifics represent up to 4% of all the prey items ingested by these elapids, and that this behaviour might have led to young specimens feeding in different areas to adults to avoid predation (Maritz, Alexander and Maritz, 2019).

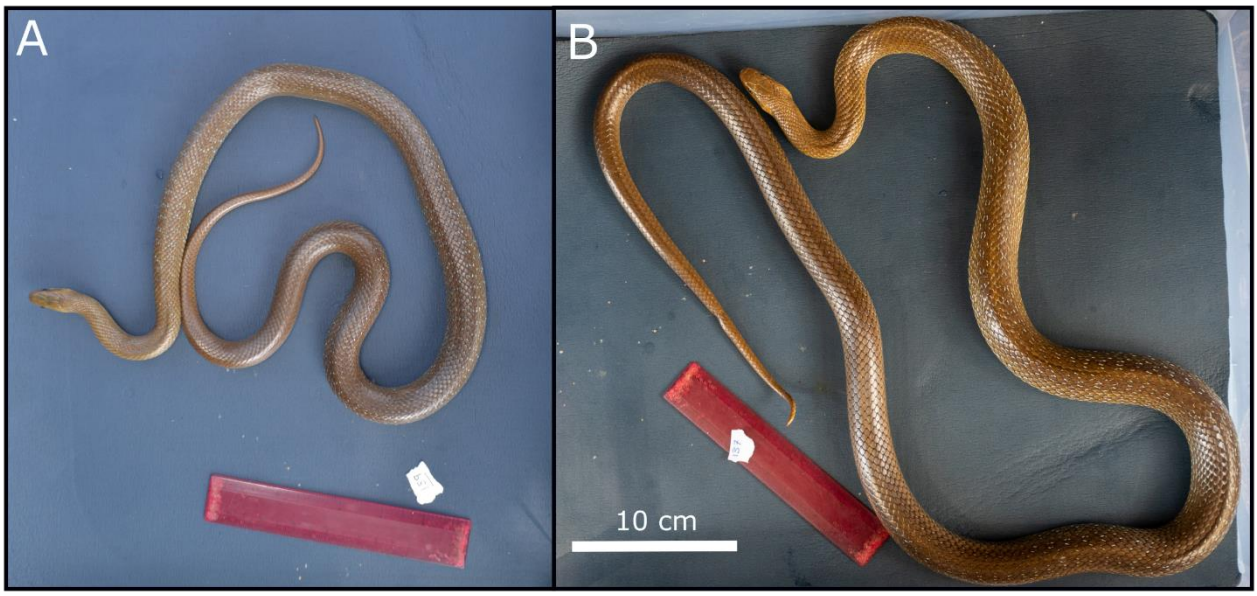

Figure 1: The female Aesculapian snake, *Zamenis longissimus* (A) was eaten by the male (B).

The reasons underlying the observed behaviour may be various. Firstly, cannibalistic behaviours are often correlated with the decline or absence of suitable prey, especially in the case of generalist predators (Polis, 1981, Jofré and Reading, 2020). It is possible that there is limited prey availability in the restricted range of Welsh Aesculapian snakes, although we would expect a healthy population of rodents due to the close proximity to the Welsh Mountain Zoo, where lots of animal feed is present. Cannibalism among snakes may also be associated with high population density, where the high encounter rate among conspecifics could favour cannibalism (Faraone, Di Nicola and Lo Valvo, 2020).

We strongly suspect that the cannibalised female laid eggs before the event, for a few reasons. Firstly, she was coiled up with a male snake when initially captured, suggesting she may have mated. She also made an uncharacteristic long movement during July which we interpret as finding a suitable egg-laying site. This was reinforced by her apparent weight loss immediately

afterwards. Therefore, it is plausible that the potentially emaciated and smaller female simply represented a significant source of energy intake for the male. Moreover, *Z. longissimus* is characterised by a strong sexual dimorphism, with males growing significantly larger than females (Corti et al, 2011; Kreiner, 2007). Taking this trait into consideration, our observation fits into an asymmetric cannibalism context, with cannibalism frequently exhibited by relatively large individuals in populations with high size variance (Polis, 1981).

Cannibalism is a complex behavioural trait whose underlying causation may be hard to explain. In this case, in a very small introduced range of only a few square kilometres, bounded by roads and housing estates, it seems likely that encounter rates between individuals would be high, increasing the potential for cannibalism. Further telemetric study of this species and others may reveal that male-female cannibalism is less rare than we think.

Acknowledgements

This work was supported by the Knowledge Economy Skills Scholarships (KESS II – case number 80815) and the Welsh Mountain Zoo. We are very grateful to the numerous keepers and other staff of the Welsh Mountain Zoo for their continued help, support, and advice. This work took place with approval from the Bangor University Animal Welfare and Ethics Review Board. Telemetry procedures were conducted under Home Office project licence PC44793B6.

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